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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 6 : C12Q 1/68 | A3 | (11) International Publication Number: WO 99/55913<br>(43) International Publication Date: 4 November 1999 (04.11.99) |
|---|---|---|

(21) International Application Number: PCT/US99/09119

(22) International Filing Date: 27 April 1999 (27.04.99)

(30) Priority Data:

| | | |
|---|---|---|
| 60/083,331 | 27 April 1998 (27.04.98) | US |
| 60/098,070 | 27 August 1998 (27.08.98) | US |
| 60/118,624 | 4 February 1999 (04.02.99) | US |
| Not furnished | 27 April 1999 (27.04.99) | US |

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(88) Date of publication of the international search report: 20 January 2000 (20.01.00)

(54) Title: REDUCED COMPLEXITY NUCLEIC ACID TARGETS AND METHODS OF USING SAME

(57) Abstract

The invention provides a method of measuring the level of two or more nucleic acid molecules in a target by contacting a probe with a target comprising two or more nucleic acid molecules, wherein the nucleic acid molecules are arbitrarily sampled and wherein the arbitrarily sampled nucleic acid molecules comprise a subset of the nucleic acid molecules in a population of nucleic acid molecules; and detecting the amount of specific binding of the target to the probe. The invention also provides a method of measuring the level of two or more nucleic acid molecules in a target by contacting a probe with a target comprising two or more nucleic acid molecules, wherein the nucleic acid molecules are statistically sampled and wherein the statistically sampled nucleic acid molecules comprise a subset of the nucleic acid molecules in a population of nucleic acid molecules; and detecting the amount of specific binding of the target to the probe.



*(Referred to in PCT Gazette No. 13/2000, Section II)

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# REDUCED COMPLEXITY NUCLEIC ACID TARGETS AND METHODS OF USING SAME

This invention was made with government support under grant number CA68822, NS33377, AI34829 awarded by the National Institutes of Health and under grant number BC961294 awarded by the Department of Defense. The government has certain rights in the invention.

## BACKGROUND OF THE INVENTION

The present invention relates generally to methods of measuring nucleic acid molecules in a target and more specifically to methods of detecting differential gene expression.

Every living organism requires genetic material, deoxyribonucleic acid (DNA), which contains genes that impart a unique collection of characteristics to the organism. DNA is composed of two strands of complementary sequences of nucleotide building blocks. The two strands bind, or hybridize, with the complementary sequence to form a double helix. Genes are discreet segments of the DNA and provide the information required to generate a new organism and to give that organism its unique characteristics. Even simple organisms, such as bacteria, contain thousands of genes, and the number is many fold greater in complex organisms such as humans. Understanding the complexities of the development and functioning of living organisms requires knowledge of these genes.

For many years, scientists have searched for and identified a number of genes important in the development and function of living organisms. The search

for new genes has greatly accelerated in recent years due to directed projects aimed at identifying genetic information with the ultimate goal being the determination of the entire genome of an organism and its encoded genes, termed genomic studies. One of the most ambitious of these genomic projects has been the Human Genome Project, with the goal of sequencing the entire human genome. Recent advances in sequencing technology have led to a rapid accumulation of genetic information, which is available in both public and private databases. These newly discovered genes as well as those genes soon to be discovered provide a rich resource of potential targets for the development of new drugs.

Despite the rapid pace of gene discovery, there remains a formidable task of characterizing these genes and determining the biological function of these genes. The characterization of newly discovered genes is often a time consuming and laborious undertaking, sometimes taking years to determine the function of a gene or its gene product, particularly in complex higher organisms.

Another level of complexity arises when complex interactions between genes and their gene products are contemplated. To understand how an organism works, it is important not only to understand what role a gene, its transcript and its gene product plays in the workings of an organism, it is also important to understand potentially complex interactions between the gene, its transcript, or its gene product and other genes and their gene products.

A number of approaches have been used to assess gene expression in a particular cell or tissue of an organism. These approaches have been used to

characterize gene expression under various conditions, including looking at differences in expression under differing conditions. However, most of these methods are useful for detecting transcripts that are abundant transcripts but have proven less useful for detecting transcripts that are of low abundance, particularly when looking at the expression of a number of genes rather than a selected few genes. Since genes expressed at low levels often regulate the physiological pathways in a cell, it is desirable to detect transcripts having at low abundance.

Thus, a need exists for a method to characterize the expression pattern of genes under a given set of conditions and to detect low abundance transcripts. The present invention satisfies this need and provides related advantages as well.

## SUMMARY OF THE INVENTION

The invention provides a method of measuring the level of two or more nucleic acid molecules in a target by contacting a probe with a target comprising two or more nucleic acid molecules, wherein the nucleic acid molecules are arbitrarily sampled and wherein the arbitrarily sampled nucleic acid molecules comprise a subset of the nucleic acid molecules in a population of nucleic acid molecules; and detecting the amount of specific binding of the target to the probe. The invention also provides a method of measuring the level of two or more nucleic acid molecules in a target by contacting a probe with a target comprising two or more nucleic acid molecules, wherein the nucleic acid molecules are statistically sampled and wherein the statistically sampled nucleic acid molecules comprise a

subset of the nucleic acid molecules in a population of nucleic acid molecules; and detecting the amount of specific binding of the target to the probe.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows differential hybridization to clone arrays. Each image is an autoradiogram that spans about 4000 double spotted *E. coli* colonies, each carrying a different EST clone. Panel A shows the binding of a total target made from 1 µg of polyA$^+$ RNA from confluent human keratinocytes that was radiolabeled during reverse transcription. Panels B and C show RAP-PCR fingerprint with a pair of arbitrary primers that was performed on cDNA from oligo(dT) primed cDNA of confluent human keratinocytes that were untreated (Panel B) and treated with epidermal growth factor (EGF) (Panel C). The two radiolabeled colonies from one differentially expressed cDNA are indicated with an arrow. Panel D shows a RAP-PCR fingerprint with a different pair of arbitrary primers that was performed on RNA from confluent human keratinocytes.

Figure 2 shows RAP-PCR fingerprints resolved on a polyacrylamide-urea gel. Reverse transcription was performed with an oligo-dT primer on 250, 125, 62.5 and 31.25 ng RNA in lanes 1, 2, 3, and 4 respectively. RNA was from untreated, TGF-β and EGF treated HaCaT cells, as indicated. RAP-PCR was performed with two sets of primers, primers GP14 and GP16 (Panel A) or Nuc1+ and OPN24 (Panel B). Molecular weight markers are indicated on the left of each panel, and the sizes of the two differentially amplified RAP-PCR-products are indicated with arrows (317 and 291).

Figure 3 shows hybridization of targets generated by RAP-PCR to arrays. Shown are autoradiograms of the bottom half of duplicates of the same filter (Genome Systems) hybridized with radiolabeled DNA. Panels A and B show hybridization of two RAP-PCR reactions generated using the same primers and derived from untreated (Panel A) and EGF treated (Panel B) HaCaT cells. Three double-spotted clones that show differential hybridization signals are marked on each array. The GenBank accession numbers of the clone and the corresponding genes are H10045 and H10098, corresponding to *vav-3* and AF067817 (square); H28735, gene unknown, similar to $heparan sulfate 3-O-sulfotransferase-1, AF019386 (circle); R48633, gene unknown (diamond). Panel C shows an array hybridized with a RAP-PCR target generated using the same RNA as in panel A but with a different pair of primers. Panel D shows an array hybridized with cDNA target generated by reverse transcription of 1 μg poly(A)$^+$-selected mRNA. Panel E shows an array hybridized with human genomic DNA labeled using random priming.

Figure 4 shows resolution of RT-PCR products on polyacrylamide-urea gels and confirmation of differential regulation in response to EGF using low stringency RT-PCR. Reverse transcription was performed at two RNA concentrations (500 ng, left column; 250 ng, right column) at different cycle numbers. Shown are bands for the control (22 cycles); for GenBank accession number H11520 (22 cycles); for TSC-22, corresponding to GenBank accession numbers H11073 and H11161 (19 cycles); and for R48633 (19 cycles).

Figure 5 shows differential display of untreated and EGF treated HaCaT cells. Panel A shows

differential display reactions performed at four different starting concentrations of total RNA (designated 1, 2, 3 and 4 and corresponding to 800, 400, 200 and 100 ng, respectively), which was then used for PCR. An anchored oligo(dT) primer, H-$T_{11}$C or H-$T_{11}$A, was used in combination with one of two different arbitrary primers, H-AP3 or H-AP4, which are indicated above the lanes. Panel B shows differential display using the arbitrary primer KA2 with three different anchored oligo(dT) primers, $T_{13}$V, $AT_{15}$A and $GT_{15}$G, used at four different starting concentrations of RNA (designated 1, 2, 3 and 4 and corresponding to 1000, 500, 250 and 125 ng, respectively), which was then used for PCR.

Figure 6 shows hybridization of differential display reactions to cDNA arrays. Differential display products generated with the primers $GT_{15}$G and KA2 from untreated (Panel A) and EGF treated (Panel B) HaCaT cells were labeled by random priming and hybridized to cDNA arrays. A section representing less than 5% of a membrane is shown with a differentially regulated gene indicated by an arrow. Panel C shows hybridization of differential display products generated with the primers $AT_{15}$A and KA2 from untreated HaCaT cells.

Figure 7 shows confirmation of differential regulation of genes by EGF using low stringency RT-PCR. Reverse transcription was performed at twofold different RNA concentrations, and low stringency PCR was performed at different cycle numbers. The amount of input RNA used for initial first strand cDNA synthesis and used in each RAP-PCR reaction was 125 ng, left column and 250 ng, right column. The RT-PCR products from 19 cycle reactions were resolved on polyacrylamide-urea gels. Shown are the products for the control (unregulated) and

genes exhibiting ≥ 1.6-fold regulation in response to EGF, corresponding to GenBank accession numbers R72714, H14529, H27389, H05545, H27969, R73247, and H21777.

Figure 8 shows the nucleotide sequence for GenBank accession number H11520 (SEQ ID NO:1).

Figure 9 shows the nucleotide sequence for GenBank accession number H11161 (SEQ ID NO:2).

Figure 10 shows the nucleotide sequence for GenBank accession number H11073 (SEQ ID NO:3).

Figure 11 shows the nucleotide sequence for GenBank accession number U35048 (SEQ ID NO:4).

Figure 12 shows the nucleotide sequence for GenBank accession number R48633 (SEQ ID NO:5).

Figure 13 shows the nucleotide sequence for GenBank accession number H28735 (SEQ ID NO:6).

Figure 14 shows the nucleotide sequence for GenBank accession number AF019386 (SEQ ID NO:7).

Figure 15 shows the nucleotide sequence for GenBank accession number H25513 (SEQ ID NO:8).

Figure 16 shows the nucleotide sequence for GenBank accession number H25514 (SEQ ID NO:9).

Figure 17 shows the nucleotide sequence for GenBank accession number M13918 (SEQ ID NO:10).

Figure 18 shows the nucleotide sequence for GenBank accession number H12999 (SEQ ID NO:11).

Figure 19 shows the nucleotide sequence for GenBank accession number H05639 (SEQ ID NO:12).

Figure 20 shows the nucleotide sequence for GenBank accession number L49207 (SEQ ID NO:13).

Figure 21 shows the nucleotide sequence for GenBank accession number H15184 (SEQ ID NO:14).

Figure 22 shows the nucleotide sequence for GenBank accession number H15124 (SEQ ID NO:15).

Figure 23 shows the nucleotide sequence for GenBank accession number X79781 (SEQ ID NO:16).

Figure 24 shows the nucleotide sequence for GenBank accession number H25195 (SEQ ID NO:17).

Figure 25 shows the nucleotide sequence for GenBank accession number H24377 (SEQ ID NO:18).

Figure 26 shows the nucleotide sequence for GenBank accession number M31627 (SEQ ID NO:19).

Figure 27 shows the nucleotide sequence for GenBank accession number H23972 (SEQ ID NO:20).

Figure 28 shows the nucleotide sequence for GenBank accession number H27350 (SEQ ID NO:21).

Figure 29 shows the nucleotide sequence for GenBank accession number AB000712 (SEQ ID NO:22).

Figure 30 shows the nucleotide sequence for GenBank accession number R75916 (SEQ ID NO:23).

Figure 31 shows the nucleotide sequence for GenBank accession number X85992 (SEQ ID NO:24).

Figure 32 shows the nucleotide sequence for GenBank accession number R73021 (SEQ ID NO:25).

Figure 33 shows the nucleotide sequence for GenBank accession number R73022 (SEQ ID NO:26).

Figure 34 shows the nucleotide sequence for GenBank accession number U66894 (SEQ ID NO:27).

Figure 35 shows the nucleotide sequence for GenBank accession number H10098 (SEQ ID NO:28).

Figure 36 shows the nucleotide sequence for GenBank accession number H10045 (SEQ ID NO:29).

Figure 37 shows the nucleotide sequence for GenBank accession number AF067817 (SEQ ID NO:30).

Figure 38 shows the nucleotide sequence for GenBank accession number R72714 (SEQ ID NO:31).

Figure 39 shows the nucleotide sequence for GenBank accession number X52541 (SEQ ID NO:32).

Figure 40 shows the nucleotide sequence for GenBank accession number H14529 (SEQ ID NO:33).

Figure 41 shows the nucleotide sequence for GenBank accession number M10277 (SEQ ID NO:34).

Figure 42 shows the nucleotide sequence for GenBank accession number H27389 (SEQ ID NO:35).

Figure 43 shows the nucleotide sequence for GenBank accession number D89092 (SEQ ID NO:36).

Figure 44 shows the nucleotide sequence for GenBank accession number D89678 (SEQ ID NO:37).

Figure 45 shows the nucleotide sequence for GenBank accession number H05545 (SEQ ID NO:38).

Figure 46 shows the nucleotide sequence for GenBank accession number J03804 (SEQ ID NO:39).

Figure 47 shows the nucleotide sequence for GenBank accession number H27969 (SEQ ID NO:40).

Figure 48 shows the nucleotide sequence for GenBank accession number R73247 (SEQ ID NO:41).

Figure 49 shows the nucleotide sequence for GenBank accession number U51336 (SEQ ID NO:42).

Figure 50 shows the nucleotide sequence for GenBank accession number H21777 (SEQ ID NO:43).

Figure 51 shows the nucleotide sequence for GenBank accession number K00558 (SEQ ID NO:44).

Figure 52 shows the nucleotide sequence for GenBank accession number D31765 (SEQ ID NO:45).

## DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods for measuring the level of two or more nucleic acid molecules in a target by contacting a probe with an arbitrarily sampled target or a statistically sampled target and detecting the amount of specific binding to the probe. The invention also provides methods of identifying two or more differentially expressed nucleic acid molecules associated with a condition by measuring the level of two or more nucleic acid molecules in a target and comparing the expression levels to expression levels of the nucleic acid molecules in a second target. The methods of the invention are useful for obtaining a profile of nucleic acid molecules expressed in a target under a given set of conditions. The methods of the invention are particularly useful for comparing the relative abundance of low abundance nucleic acid molecules between two or more targets. The methods of the invention are advantageous in that a profile of nucleic acid molecule abundance can be determined and correlated with a given set of conditions or compared to another target to determine if the original target was exposed to a particular set of conditions, thereby providing information useful for assessing the diagnosis or treatment of a disease.

The invention provides a method of measuring the abundance of two or more nucleic acid molecules in a target. The method of the invention includes the steps of contacting a probe with a target comprising two or more nucleic acid molecules, wherein the nucleic acid molecules are arbitrarily sampled and wherein the arbitrarily sampled nucleic acid molecules comprise a subset of the nucleic acid molecules in a population of

nucleic acid molecules; and detecting the amount of specific binding of the target to the probe.

As used herein, the term "nucleic acid molecule" refers to a nucleic acid of two or more nucleotides. A nucleic acid molecule can be RNA or DNA. For example, a nucleic acid molecule can include messenger RNA (mRNA), transfer RNA (tRNA) or ribosomal RNA (rRNA). A nucleic acid molecule can also include, for example, genomic DNA or cDNA. A nucleic acid molecule can be synthesized enzymatically, either *in vivo* or *in vitro*, or the nucleic acid molecule can be chemically synthesized by methods well known in the art. A nucleic acid molecule can also contain modified bases, for example, the modified bases found in tRNA such as inosine, methylinosine, dihyrouridine, ribothymidine, pseudouridine, methylguanosine and dimethylguanosine. Furthermore, a chemically synthesized nucleic acid molecule can incorporate derivatives of nucleotide bases.

As used herein, the term "population of nucleic acid molecules" refers to a group of two or more different nucleic acid molecules. A population of nucleic acid molecules can also be 3 or more, 5 or more, 10 or more, 20 or more, 50 or more, 100 or more, 1000 or more or even 10,000 or more different nucleic acid molecules. The nucleic acid molecules can differ, for example, by a single nucleotide or by modification of a single base. Generally, a population of nucleic acid molecules is obtained from a target sample, for example, a cell, tissue or organism. In such a case, the population of nucleic acid molecules contains the nucleic acid molecules of the target sample.

A population of nucleic acid molecules has characteristics that can differentiate one population of nucleic acid molecules from another. These characteristics are based on the number and nature of individual nucleic acid molecules comprising the population. Such characteristics include, for example, the abundance of nucleic acid molecules in the population. The abundance of an individual nucleic acid molecule can be an absolute amount in a given target sample or can be the amount relative to other nucleic acid molecules in the target sample. In a population of nucleic acid molecules obtained from a target, individual nucleic acid molecules can be more abundant or less abundant relative to other nucleic acid molecules in the sample target. A less abundant sequence can also be relative abundance between two samples.

As used herein, a less abundant nucleic acid molecule can be, for example, less than about 10% as abundant as the most abundant nucleic acid molecule in a population. A less abundant nucleic acid molecule can also be less than about 1% as abundant, less than about 0.1% as abundant or less than about 0.01% as abundant as the most abundant nucleic acid molecule in a population. For example, a low abundance nucleic acid molecule can be less than about 10 copies per cell, or even as low as 1 copy per cell.

Another characteristic of a population of nucleic acid molecules is the complexity of the population. As used herein, "complexity" refers to the number of nucleic acid molecules having different sequences in the population. For example, a population of nucleic acid molecules representative of the mRNA in a bacterial cell has lower complexity than a population of

nucleic acid molecules representative of the mRNA in a eukaryotic cell, a tissue or an organism because a smaller number of genes are expressed in a bacterial cell relative to a eukaryotic cell, tissue or organism.

A population of nucleic acid molecules can also be characterized by the properties of individual nucleic acid molecules in the population. For example, the length of individual nucleic acid molecules contributes to the characteristics of a population of nucleic acid molecules. Similarly, the sequence of individual nucleic acid molecules in the population contributes to the characteristics of the population of nucleic acid molecules, for example, the G+C content of the nucleic acid sequences and any secondary structure that can form due to complementary stretches of nucleotide sequence that can undergo intrastrand hybridization.

As used herein, the term "subset of nucleic acids" means less than all of a set of nucleic acid molecules. For example, a subset of nucleic acid molecules of a target sample population would be less than all of the nucleic acid molecules in the target sample population. Specifically excluded from a subset of nucleic acid molecules is a group of nucleic acid molecules representative of all the nucleic acid molecules in a sample target, for example, a target generated using total cDNA or total mRNA.

As used herein, the term "target" refers to one or more nucleic acid molecules to which binding of a probe is desired. A target is detectable when bound to a probe. A target of the invention generally comprises two or more different nucleic acid molecules. A target can be derived from a population of nucleic acid molecules

from a cell, tissue or organism. A target can also contain 3 or more, 5 or more, 10 or more, 20 or more, 30 or more, 50 or more, 100 or more, 200 or more, 500 or more, 1000 or more, 2000 or more, 5000 or more, or even 10,000 or more different nucleic acid molecules. A target can have a detectable moiety associated with it such as a radioactive label, a fluorescent label or any label that is detectable. When a target is labeled, for example, with a radioactive label, the target can be used "to probe" or hybridize with other nucleic acid molecules. Methods of making a target are disclosed herein.

A method of detection that directly measures binding of the target to a probe, without the need for a detectable moiety attached to the target, can also be used. In such a case, the nucleic acid molecules are directly detectable without modification of a nucleic acid molecule of the target, for example, by attaching a detectable moiety. An example of such a detection method using a target without a detectable moiety is detection of binding of a target using mass spectrometry. Another example of a method using a target containing nucleic acid molecules without an attached detectable moiety is binding the target to a probe that contains molecules having a detectable moiety. In such a case, the binding of a target to the probe containing molecules having a detectable moiety is detected and, as such, the target is detectable when bound to the probe. An example is the "molecular beacon," where probe binding causes separation of a fluorescent tag from a fluorescence quencher.

As used herein, the term "specific binding" means binding that is measurably different from a non-specific interaction. Specific binding can be

measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding of a target to a probe can be determined by comparing binding of the target with binding control nucleic acids not included in the target. Specific binding can also be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of a labeled target to a probe is competitively inhibited by excess unlabeled target.

The term "specific binding," as used herein, includes both low and high affinity specific binding. Specific binding can be exhibited, for example, by a low affinity molecule having a Kd of at least about $10^{-4}$ M. Specific binding also can be exhibited by a high affinity molecule, for example, a molecule having a Kd of at least about of $10^{-7}$ M, at least about $10^{-8}$ M, at least about $10^{-9}$ M, at least about $10^{-10}$ M, or can have a Kd of at least about $10^{-11}$ M or $10^{-12}$ M or greater.

In the case of a probe comprising an array of nucleic acid molecules, binding of a specific nucleic acid molecule of the probe to another nucleic acid molecule is also known as hybridizing or hybridization. As used herein, the term "hybridizing" or "hybridization" refers to the ability of two strands of nucleic acid molecules to hydrogen bond in a sequence dependent manner. Under appropriate conditions, complementary nucleotide sequences can hybridize to form double stranded DNA or RNA, or a double stranded hybrid of RNA and DNA. Nucleic acid molecules with similar but non-

identical sequences can also hybridize under appropriate conditions.

As used herein, the term "probe" refers to a population of two or more molecules to which binding of a target is desired. The molecules of a probe include nucleic acid molecules, oligonucleotides and polypeptide-nucleic acid molecules. A probe can additionally be an array of molecules.

In general, a probe is comprised of molecules immobilized on a solid support and the target is in solution. However, it is understood that a target can be bound to a solid support and a probe can be in solution. Furthermore, both the probe and the target can be in solution. It is understood that the configuration of the probe and target can be in solution or bound to a solid support, so long as the probe and target can bind to each other. When bound to a solid support, the binding of the probe or target to the support can be covalent or non-covalent, so long as the bound probe or target remains bound under conditions of contacting the solid support with a probe or target in solution and washing of the solid support. If the probe and target hybridize or otherwise specifically interact, the probe or target bound to a solid support remains bound during the hybridization and washing steps.

As used herein, the term "sampled" or "samples," when used in reference to a nucleic acid molecule, refers to a nucleic acid molecule to which specific binding can be detected. A nucleic acid molecule that samples another molecule is capable of specifically binding to that molecule and being detected. For example, a probe can sample molecules in a target by

detectably binding to molecules in the target. Those molecules in the target to which nucleic acid molecules in the probe specifically bind are therefore sampled.

As used herein, the term "arbitrarily sampled" or "arbitrarily sampled nucleic acid molecule" means that a nucleic acid molecule is sampled by binding based on its sequence without sampling based on a particular site where a molecule will bind. When generating a target comprising arbitrarily sampled nucleic acid molecules from a population of nucleic acid molecules, the target is generated without prior reference to the sequences of nucleic acid molecules in the population. Thus, it is not necessary to have previous knowledge of the nucleotide sequence of nucleic acid molecules in the population to arbitrarily sample the population. It is understood that knowledge of a nucleotide sequence of a nucleic acid molecule in the population does not preclude the ability to arbitrarily sample the population so long as the nucleotide sequence is not referenced before sampling the population. Methods for generating a probe containing arbitrarily sampled nucleic acid molecules are disclosed herein (see below and Examples I to III).

An arbitrarily sampled probe containing arbitrarily sampled nucleic acid molecules can be generated using one or more arbitrary oligonucleotides. As used herein, the term "arbitrary oligonucleotide" means that the oligonucleotide is a sequence that is selected randomly and is not selected based on its complementarity to any known sequence. As such, an arbitrary oligonucleotide can be used to arbitrarily sample a population of nucleic acid molecules.

An arbitrarily sampled nucleic acid molecule is sampled based on its sequence and is not based on binding to a predetermined sequence. For example, arbitrary oligonucleotides are oligonucleotides having an arbitrary sequence and, as such, will bind to a given nucleic acid molecule because the complementary sequence of the arbitrary oligonucleotide occurs by chance in the nucleic acid molecule. Because the oligonucleotides can bind to a nucleic acid molecule based on the presence of a complementary sequence, the sampling of the nucleic acid molecule is based on that sequence. However, the binding of the arbitrary oligonucleotide to any particular nucleic acid molecule in a population is not determined prior to the binding of the oligonucleotide, for example, by comparing the sequence of the arbitrary oligonucleotides to known nucleic acid sequences and selecting the oligonucleotides based on previously known nucleic acid sequences. The use of arbitrary oligonucleotides as primers for amplification is well known in the art (Liang and Pardee, Science 257:967-971 (1992)).

As used herein, the term "oligonucleotide" refers to a nucleic acid molecule of at least 2 and less than about 1000 nucleotides. An oligonucleotide can be, for example, at least about 5 nucleotides and less than about 100 nucleotides, for example less than about 50 nucleotides.

The invention also provides a method of measuring the level of two or more nucleic acid molecules in a target by contacting a probe with a target comprising two or more nucleic acid molecules, wherein the nucleic acid molecules are statistically sampled and wherein the statistically sampled nucleic acid molecules

comprise a subset of the nucleic acid molecules in a population of nucleic acid molecules; and detecting the amount of specific binding of the target to the probe.

As used herein, the term "statistically sampled nucleic acid molecule" means that a nucleic acid sequence is sampled based on its sequence with prior reference to its nucleotide sequence by predetermining the statistical occurrence of a nucleotide sequence in two or more nucleic acid molecules. Thus, to obtain a statistically sampled nucleic acid molecule, it is necessary to have previous knowledge of the nucleotide sequence of at least two nucleic acid molecules in the population.

A statistically sampled nucleic acid molecule is sampled based on the sequence of a nucleic acid molecule with prior reference to its nucleotide sequence but without prior reference to a preselected portion of its nucleotide sequence. A group of oligonucleotides can be identified without prior reference to a preselected portion of a nucleotide sequence, for example, by determining a group of arbitrary oligonucleotides. The arbitrary oligonucleotides can then be referenced to known nucleotide sequences by determining which of the arbitrary primers match the known nucleotide sequences. Such arbitrary oligonucleotides referenced to known nucleotide sequences are selected based on the known sequences and thus become statistical primers. This method is in contrast to a method where a preselected site in a known nucleotide sequence is identified and an oligonucleotide is specifically designed to match that preselected site.

Statistical sampling is advantageous because a set of oligonucleotides can be determined based on the

presence in a group of known sequences of a sequence complementary to the oligonucleotides. The oligonucleotides can further be ranked based on complexity binding. Complexity binding means that a given oligonucleotide binds to more than one nucleic acid molecule. The larger the number of molecules to which an oligonucleotide can bind, the higher the "complexity binding." Statistical selection can be used to enhance for complexity binding by ranking oligonucleotides based on the number of sequences to which the oligonucleotides will bind and selecting those that bind to the highest number (see, for example, WO 99/11823). Statistical sampling can be based, for example, on the binding of an oligonucleotide to 5 or more nucleic acid molecules, and can be based on the binding to 10 or more, 50 or more, 100 or more, 200 or more, 500 or more, 1000 or more, or even 10,000 or more nucleic acid molecules.

In addition, statistical sampling can enhance for the highest complexity binding for a given oligonucleotide, for example, by selecting the above average ranked oligonucleotides that are complementary to above the average number of nucleic acid molecules. The oligonucleotides can be selected for the any range of complexity binding, for example, the top 10% of highest ranked complexity binding, the top 20% of highest ranked complexity binding, or the top 50% of highest ranked complexity binding.

Furthermore, statistical selection can be used to exclude undesirable nucleotide sequences, including conserved sequences in a family of related nucleic acid molecules (WO 99/11823). A statistical oligonucleotide can be about 5 nucleotides in length to about 1000 nucleotides in length, for example, about 5, 6, 7, 8, 9,

10, 11, 12, 13, 14, 15, 16, 18, 20, 25, 30 or 50 nucleotides in length. A set of statistical primers can contain degenerate bases, for example, more than one nucleotide at any given position.

A sampled nucleic acid molecule obtained using a preselected portion of a nucleotide sequence is specifically excluded from the meaning of the term "statistically sampled nucleic acid molecule." For example, if a portion of a known nucleotide sequence is identified and an oligonucleotide that matches the identified portion is generated to sample a nucleic acid molecule, such a sampled nucleic acid molecule would not be a statistically sampled nucleic acid molecule. However, if a group of oligonucleotides is first identified and then compared to two or more known nucleotide sequences in a population of nucleic acid molecules to determine oligonucleotides statistically present in or similar to the known nucleotide sequences, such statistically identified oligonucleotides can be used to obtain a statistically sampled nucleic acid molecule. Methods for generating a target containing statistically sampled nucleic acid molecules are disclosed herein.

A statistically sampled target containing statistically sampled nucleic acid molecules can be generated using one or more statistical oligonucleotides. As used herein, the term "statistical oligonucleotide" means that an oligonucleotide is a sequence that is selected based on its statistical occurrence of complementarity in more than one known nucleic acid molecule. As such, a statistical oligonucleotide can be used to statistically sample a population of nucleic acid molecules.

The methods of the invention detect specific binding of a target to a probe. A target can be generated, for example, by amplifying nucleic acid molecules. As used herein, the term "amplified target" refers to a target generated by enzymatically copying a nucleic acid molecule to generate more than one copy of the nucleic acid molecules in a population of nucleic acid molecules. An amplified nucleic acid target can be generated, for example, using an amplification method such as polymerase chain reaction (PCR). A target having a single copy of each nucleic acid molecule in a target sample from which the target sample is derived, which would have identical abundance and complexity as the original population, would not be considered an amplified target. An amplified target can be useful, for example, if nucleic acid molecules sampled by the probe are in limited quantities in the target. A nucleic acid molecule that is to be sampled and which is present in very low quantities would be difficult to detect without amplification and increasing the mass of the nucleic acid molecules in the probe. However, a limited complexity target, in which the complexity or number of different molecules is limited, need not be amplified.

Other methods for generating an amplified target include, for example, the ligase chain reaction (LCR); self-sustained sequence replication (3SR); beta replicase reaction, for example, using Q-beta replicase; phage terminal binding protein reaction; strand displacement amplification (SDA); nucleic acid sequence based amplification (NASBA); cooperative amplification by cross hybridization (CATCH); rolling circle amplification (RCA) and AFLP (Trippler et al., J. Viral. Hepat. 3:267 (1996); Hofler et al., Lab. Invest. 73:577 (1995); Tyagi et al., Proc. Natl. Acad. Sci. USA 93:5395 (1996); Blanco

et al., Proc. Natl. Acad. Sci. USA 91:12198 (1994); Spears et al., Anal. Biochem. 247:130 (1997); Spargo et al., Mol. Cell. Probes 10:247 (1996); Gobbers et al., J. Virol. Methods 66:293 (1997); Uyttendaele et al., Int. J. Food Microbiol. 37:13 (1997); and Leone et al., J. Virol. Methods 66:19 (1997); Ellinger et al., Chem. Biol. 5:729-741 (1998); Ehricht et al., Nucleic Acids Res. 25:4697-4699 (1997); Ehricht et al., Eur. J. Biochem. 243:358-364 (1997); Lizardi et al., Nat. Genet. 19:225-232 (1998)).

The methods of the invention are useful for measuring the level of two or more nucleic acid molecules in a target. The methods of the invention can also be used to compare expression levels between two targets. In particular, the methods of the invention are useful for measuring differential expression of nucleic acid molecules (see below).

A total target, using the full complexity of the mRNA population for target preparation, can easily examine the top few hundred or a few thousand of the mRNAs in the cell (Pietu et al., Genome Res. 6:492-503 (1996)). However, a total labeled cDNA target from a mammalian cell typically has a complexity of over 100 million bases which complicates attempts to detect differential expression among the rarer mRNAs using differential hybridization. Recent advances in the use of fluorescence and confocal microscopy have led to improvements in the sensitivity and dynamic range of differential hybridization methods, with a dynamic range of detection of 10,000-fold and the detection of transcripts at a sensitivity approaching 1/500,000 (Marshall and Hodgson, Nat. Biotechnol. 16:27-31 (1998); Ramsay, Nat. Biotechnol. 16:40-44 (1998)). Despite the

improvements in sensitivity, methods using total target remain biased toward more abundant mRNAs in a sample.

The standard method for differential screening, which typically uses targets derived from reverse transcription of total message and autoradiography or phosphoimaging, can be used to detect differential expression (Pietu, *supra*, 1996). However, the method is limited to the most abundant messages. Only abundant transcripts are represented highly enough to yield effective targets with a sensitivity of perhaps 1/15,000 (Boll, Gene 50:41-53 (1986)). As disclosed herein, differential screening can be improved greatly by reducing the complexity of the target and by systematically increasing the amount of rarer nucleic acid molecules in the target. By enhancing the amount of less abundant nucleic acids in a target, differential screening is not confined to only the most abundant nucleic acid molecules, as observed using total target.

By reducing the complexity of the target, the ability to identify all mRNA species in a source simultaneously is sacrificed for improved kinetics and an improved signal to noise ratio. Complexity reduction methods generate a target having a subset of nucleic acid molecules in a population that allow a few rare mRNAs to contribute significantly to the final mass of the target, thereby enhancing the ability to observe differential gene expression among rare mRNAs in a source. Any method that generates a mixture of products that reliably enriches for only part of each mRNA or only a subset of the mRNA population is useful for generating a reduced complexity target.

There are two fundamentally different types of complexity reduction methods, methods that maintain the relative stoichiometry among the mRNAs they sample and methods that do not maintain stoichiometry. One class of methods yields nucleic acids representing a subset of the mRNA population and maintains the approximate stoichiometry of the input RNA. Such methods are exemplified by most amplified restriction fragment length polymorphism (AFLP) and restriction strategies that sample the 3′ end or internal fragments of mRNAs (Habu et al., Biochem. Biophys. Res. Commun. 234:516-521 (1997); Money et al., Nucleic Acids Res. 24:2616-2617 (1996); Bachem et al., Plant J. 9:745-753 (1996)). Another example is the use of size fractionated mRNAs to generate cDNA targets. All the mRNAs, for example, the 2.0 to 2.1 kb range can be used as a reduced complexity target. Stoichiometry among these mRNAs would be mostly preserved in the target (Dittmar et al., Cell Biol. Int. 21:383-391 (1997)).

A second class of methods for generating reduced complexity targets does not preserve the stoichiometry of the starting mRNAs, though it does preserve differences among individual RNAs between target samples from which targets are made. One method to generate a reduced complexity target that does not maintain stoichiometry is to use subtracted targets, which have shown sensitivity for rare messages comparable to chips, in particular methods based on representational difference analysis or suppression subtractive hybridization (Rhyner et al., J. Neurosci. Res. 16:167-181 (1986); Lisitsyn et al., Science 259:946-951 (1993); Lisitsyn & Wigler, Methods Enzymol. 254:291-304 (1995); Jin et al., Biotechniques 23:1084-1086 (1997)).

Particularly useful methods for generating a reduced complexity target that does not maintain stoichiometry are exemplified by using arbitrarily sampled targets or statistically sampled targets. Methods using arbitrarily sampled targets and statistically sampled targets are disclosed herein. The methods using arbitrarily sampled or statistically sampled targets allow detection of low abundance nucleic acid molecules in a target. The methods of the invention are advantageous because they enhance the ability to detect low abundance nucleic acid molecules in a target and also allow detection of nucleic acid molecules in a target derived from limited quantities of nucleic acid molecules, such as a few cells or even a single cell.

An arbitrarily sampled target or statistically sampled target can be generated, for example, by amplification. If an amplified target is generated using arbitrary oligonucleotides or statistical oligonucleotides, the amplified products reflect a function of both the starting abundance of each target nucleic acid molecule and the quality of the match of the oligonucleotide to the target nucleic acid molecule to be sampled. Thus, the final mixture of amplified products can include quite abundant amplified products that derive from low abundance nucleic acid molecules that have a good match with the oligonucleotide primers used and have favorable "amplifiability" after the initial priming events. Amplifiability includes effects such as secondary structure and product size.

A consequence of generating an amplified target using arbitrary oligonucleotides or statistical oligonucleotides is that the same nucleic acid molecules in two different targets experience an identical

combination of primability and amplifiability so that changes in abundance for particular mRNAs are maintained, even as the relative abundances between different nucleic acid molecules within one target are profoundly changed. This is in contrast to methods that maintain stoichiometry, where less abundant nucleic acid molecules would be present as less abundant nucleic acid molecules in the target.

When generating an amplified target, there are generally no particular constraints on the oligonucleotide primers. The oligonucleotide primers preferably contain at least a few C or G bases. The oligonucleotide primers also preferably do not contain 3' ends complementary with themselves or the other primer in the reaction, to avoid primer dimers. The oligonucleotide primers are also preferably chosen to have different sequences so that the same parts of mRNA are not amplified in different fingerprints.

As disclosed herein, methods of generating arbitrarily sampled targets or statistically sampled targets can be based on methods that have been traditionally used to "fingerprint" a target sample containing nucleic acid molecules. The fingerprints are characteristic of the expression of nucleic acid molecules in a target sample. To generate an arbitrarily sampled target, one method that can be used is based on RNA arbitrarily primed PCR (RAP-PCR) (see Examples I and II; Welsh et al., Nucleic Acids Res. 18:7213-7218 (1990); Welsh et al., Nucleic Acids Res. 20:4965-4970 (1992); Liang and Pardee, Science 257:967-971 (1992)).

In RAP-PCR, both the abundance and the extent of match with the primers contribute to the prevalence of

any particular product. Thus, rare mRNAs that happen to have excellent matches with the primers and are efficiently amplified are found among the more abundant RAP-PCR products, which makes a target generated by RAP-PCR non-stoichiometric. This is a very useful feature of RAP-PCR because it allows the sampling of mRNAs that are difficult to sample using other methods.

In a typical RAP-PCR fingerprint, about 50-100 cDNA fragments per lane are visible on a polyacrylamide gel, including products from relatively rare mRNAs that happen to have among the best matches with the arbitrary primers. If only 100 cDNA clones could be detected in an array by each target, then hybridization to arrays would be inefficient. However, RAP-PCR fingerprints contain many products that are too rare to visualize by autoradiography of a polyacrylamide gel. Nonetheless, these rarer products are reproducible and of sufficient abundance to serve as target for arrays when labeled at high specific activity.

As disclosed herein, a single target derived from RAP-PCR can detect about a thousand cDNAs on an array containing about 18,000 EST clones, a 10-20 fold improvement over the performance of fingerprints displayed on denaturing polyacrylamide gels. In addition, when a differentially regulated gene is detected on a cDNA array, a clone representing the transcript is immediately available, and often sequence information for the clone is also available. Furthermore, the clones are usually much longer than the usual RAP-PCR product. In contrast, the standard approaches to RNA fingerprinting require that the product be gel purified and sequenced before verification of differential expression can be performed. As disclosed

herein, differentially amplified RAP-PCR products that are below the detection capabilities of the standard denaturing polyacrylamide gel and autoradiography methods can be detected using hybridization to cDNA arrays.

An arbitrarily sampled target generated by RAP-PCR can sample the top few thousand highest expressed nucleic acid molecules in a target sample and can sample different subsets of the nucleic acid molecules in a population, depending on the oligonucleotide primers used for amplification. Some of the rare nucleic acid molecules in a target are sufficiently represented to be easily detected on arrays of colonies (see Examples I and II).

To generate an arbitrarily sampled target using RAP-PCR, the RAP-PCR fingerprint is made by arbitrarily primed reverse transcription and PCR of nucleic acid molecules in a target sample, for example, messenger RNA (McClelland et al., in <u>Differential Display Methods and Protocols</u>, Liang and Pardee, eds., Humana Press (1997)). Alternatively, first strand cDNA can be primed with oligo dT or with random short oligomers, followed by arbitrary priming. Analysis of such a RAP-PCR "fingerprint" by gel electrophoresis reveals a complex fingerprint showing relative abundances of an arbitrary sample of about 100 transcripts (see Example II).

As disclosed herein, RAP-PCR fingerprints were converted to targets to probe or hybridize human cDNA clones arrayed as *E. coli* colonies on nylon membranes (Example II). Each array contained 18,432 cDNA clones from the Integrated Molecular Analysis of Genomes and their Expression (I.M.A.G.E.) consortium. Hybridization to about 1000 cDNA clones was detected using each

arbitrarily sampled target generated by RAP-PCR. Different RAP-PCR fingerprints gave hybridization patterns having very little overlap (<3%) with each other, or with hybridization patterns from total cDNA targets. Consequently, repeated application of RAP-PCR targets allows a greater fraction of the message population to be screened on this type of array than can be achieved with a radiolabeled total cDNA target.

The arbitrarily sampled targets were generated from HaCaT keratinocytes treated with EGF. Two RAP-PCR targets hybridized to 2000 clones, from which 22 candidate differentially expressed genes were observed (Example II). Differential expression was tested for 15 of these clones using RT-PCR and 13 were confirmed. The use of this cDNA array to analyze RAP-PCR fingerprints allowed for an increase in detection of 10- to 20-fold over the conventional denaturing polyacrylamide gel approach to RAP-PCR or differential display. Throughput is vastly improved by the reduction in cloning and sequencing afforded by the use of arrays. Also, repeated cloning and sequencing of the same gene, or of genes already known to be regulated in the system of interest, is minimized.

The use of RAP-PCR to generate an arbitrarily sampled target is particularly useful because it allows very high throughput discovery of differentially regulated genes (see Examples II and III). The throughput using this method is about 20 times faster. Essentially, once a RAP-PCR fingerprint has been generated, instead of analyzing the product by gel electrophoresis, the RAP-PCR fingerprint is used as a target to probe or hybridize to nucleic acid molecules.

Such an arbitrarily sampled target generated by RAP-PCR is particularly useful as a target for an array.

Parameters of the RAP-PCR reaction can be varied, for example, to optimize complexity of the target and enhance complexity binding. For example, to increase the complexity, Taq polymerase Stoffel fragment, which is more promiscuous than AMPLITAQ, can be used for amplification. The oligonucleotide primers used herein (Example II) were 10 or 11 bases in length and were not degenerate, having a single base at each position. Longer oligonucleotide primers used at the same temperature can give a more complex product, as would primers with some degeneracy. However, the greater the complexity of the target, the more closely it will resemble a total mRNA target, which loses the advantage of non-stoichiometric sampling. To further vary RAP-PCR parameters, the oligonucleotide primer length, degeneracy, and 3' anchoring can be varied in the reverse transcription and PCR reactions. Various different polymerases can also be used.

The RAP-PCR fingerprint can be radiolabeled or labeled with fluorescent dyes, as described below, and used as a target to probe against dense arrays such as arrays of cDNA clones. Differences in the level of nucleic acid molecules between two targets can indicate, for example, differences in mRNA transcript levels, which usually reflects differences in gene expression levels. Differences in expression can also reflect degradation or post-translational processsing. Using an arbitrarily sampled target, each target is estimated to allow the detection of roughly 10% of the total complexity of the message population, and most importantly, this 10% very effectively includes the rare message class. The rare

message class is included in the target because, while RAP-PCR reflects message abundance between target samples, the cDNAs selected for amplification in any particular RAP-PCR reaction is determined by sequence rather than abundance. When the sequence match between oligonucleotide primers and nucleic acid molecules is very good, even if the nucleic acid molecule is in low abundance, the low abundance nucleic acid molecules have a good chance of having a larger amount of the less abundant nucleic acid molecule relative to more abundant nucleic acid molecules in the final target.

To be suitable for either gel- or array-based analysis, RAP-PCR fingerprints should remain almost identical over an eight-fold dilution of the input RNA. Low quality RAP-PCR fingerprints are usually the consequence of poor control over RNA quality and concentration. Before proceeding with the array hybridization steps, the quality of the RAP-PCR products can be verified. Because the array method has such high throughput, this extra step is neither costly, nor time-consuming, and can greatly improve efficiency by reducing the number of false positives due to poor fingerprint reproducibility. The reproducibility of RAP-PCR fingerprints as targets is exemplified herein (see Example II).

The enhanced ability of the methods of the invention to detect low abundance nucleic acid molecules in a target sample provides a major improvement over previously used methods that have limited ability to detect rare messages. It is likely that the entire complexity of the message population of a cell could be examined in a short period of time, for example, in a few weeks.

For example, as disclosed in Example II, targets generated by RAP-PCR sample a population of mRNAs largely independent of message abundance. This is because the low abundance class of messages has much higher complexity than the abundant class, making it more likely that the arbitrary primers will find good matches. Unlike differential display, RAP-PCR demands two arbitrary priming events, possibly biasing RAP-PCR toward the complex class. It is likely that the majority of the mRNA population in a cell (< 20,000 mRNAs) can be found in as few as ten RAP-PCR fingerprints.

In addition to using RAP-PCR, differential display can also be used to generate an arbitrarily sampled target (see Example III). For differential display, first, reverse transcription uses a 3' anchored primer such as an oligo(dT) primer. Next, second strand cDNA is primed with an arbitrary primer. Then PCR takes place between the arbitrary primer and the 3' anchor.

As disclosed in Example III, a combination of one arbitrary and one oligo(dT) anchor primer was used to generate an arbitrarily sampled target for cDNA arrays. Both the RAP-PCR and differential display approaches to target preparation can use less than 1/200th of the amount of RNA used in some other array hybridization methods. Each fingerprint detected about 5-10% of the transcribed mRNAs, sampled almost independent of abundance, using inexpensive *E. coli* colony arrays of EST clones. The differential display protocol was modified to generate a sufficient mass of PCR products for use as a target to probe nucleic acid molecules. The use of different oligo(dT) anchor primers with the same arbitrary primer resulted in considerable overlap among the genes sampled by each target. Overlap of sampled

genes can be avoided by using different arbitrary primers with each oligo(dT) anchor primer. Four genes not previously known to be regulated by EGF and three genes known to be regulated by EGF in other cell types were characterized using the arbitrarily sampled targets generated by differential display. The use of arbitrarily sampled targets generated by differential display is particularly useful for identification of differentially regulated genes.

A very large number of fingerprints that have been previously generated can be converted to effective targets to be probed by nucleic acid molecule arrays if the mass is increased by performing PCR on an aliquot of each fingerprint in the presence of sufficient dNTPs (100 $\mu$M) and primers (about 1 $\mu$M). Fingerprints can be reamplified, as previously shown (Ralph et al. Proc. Natl. Acad. Sci. USA 90:10710-10714 (1993)). Thus, previously determined differential display samples can be used to generate targets to probe arrays, allowing additional information to be obtained.

As disclosed herein, differential display was used to generate targets based on the method of Liang and Pardee (*supra*, 1992). The use of targets derived from oligo(dT) anchoring has some potential advantages for certain types of arrays. For example, some arrays are generated by oligo(dT) primed reverse transcription, and these clones are 3' biased. A target generated by an oligo(dT) anchored primer and an arbitrary primer should also be 3' biased so that each PCR product can hybridize to the corresponding 3' biased clone. In contrast, a target generated using arbitrary priming can sample regions internal to mRNAs. If the arbitrary product is

located further 5' in the mRNA than the 3' truncated clone, the target cannot bind to the corresponding mRNA.

Arbitrarily sampled targets generated using differential display with 3' anchored oligonucleotide primers are particularly useful for probing 3' biased libraries and, in particular, 3' biased ESTs. 3' anchoring is not useful for sampling RNAs that do not have poly(A) tails, such as most bacterial RNAs. Targets generated using 3' anchor primers would also not be suitable for PCR arrays based on internal products. 3' biased targets are also less useful for random primed libraries.

Other methods for generating an arbitrarily sampled target can also be used. One such method is a variant of RAP-PCR, called complexity limited arbitrary sample sequencing (CLASS). CLASS was conceived as a solution to a well known and frustrating limitation of Serial Analysis of Gene Expression (SAGE) (Velculescu et al., Science 270:484-487 (1995)). SAGE is a method for generating small pieces of cDNA from two sources, linking them together, and sequencing them in large numbers. The average cell contains 200,000 mRNA transcripts, representing about 20,000 different sequences, and SAGE allows sequencing of about 40 at one time. Therefore, to compare two targets using a standard sequencing apparatus, a very large number of sequencing gels, about 100, would be required to obtain information on 400,000 mRNAs, representing 200,000 mRNAs from two populations being compared. Although the method is useful for obtaining information on expression of nucleic acid molecules, each additional RNA sample increases the number of gels needed by 50, which is very expensive and time consuming. The main problem is that all 100 gels

have to be run to have confidence in the statistics on rare messages that have changed in expression from 1 to 10 copies per cell.

To solve this problem, CLASS was devised. CLASS is similar to RAP-PCR except that the oligonucleotide primers used have degenerate 3' ends. The degeneracy causes the primers to prime often, generating short sequence tags. By choosing a short PCR extension time, the predominant products come only from a fraction of the total complexity of the mRNA, and the size of this fraction can be adjusted at will by varying the number of 3+ degenerate bases. These short tags can then be concatenated and sequenced, rapidly yielding reliable statistics on a subsample of the message complexity, similar to the ligation and sequencing strategy used in SAGE (Valculescu et al., *supra*, 1995). The CLASS products can also be used as a target to probe, for example, against arrays.

The CLASS method is advantageous because additional sets of primers having degenerate 3' ends can be generated and used to obtain a different sampling of nucleic acid molecules. This iterative approach to determining nucleic acid molecule expression provides more information about a pattern of expression in a source of nucleic acid molecules than the holistic approach of SAGE (Velculescu et al., *supra*, 1995).

In contrast to SAGE, which requires nearly complete sequencing of the 100 gels to be certain of any of the rare messages, CLASS allows nucleic acid molecule populations to be partitioned into small groups so that, with 10% of the work, confidence is generated for the results of 10% of all of the genes in the cell. With one

round of CLASS, no information is obtained on 90% of the rare messages in the first pass (10 gels), but there is high confidence in the results for 10% of the nucleic acid molecules in a target sample. The high confidence in 10% of the genes is preferable because, when hunting for differentially regulated genes, it is expected that a pattern or "type of behavior" occurs during differential gene regulation. It is seldom, if ever, that a single gene is activated without the coordinate regulation of others controlled by the same pathway. Thus, if one is seeking any one of 10 low abundance transcripts regulated, for example, by a topoisomerase inhibitor, SAGE would require running 100 sequencing gels that would yield all 10 low abundance genes. In contrast, CLASS allows running 10 gels, in one-tenth the time, to identify at least one gene, which can be sufficient to identify a pattern of gene expression. Furthermore, CLASS can be used iteratively using different primers to run additional gels, for example, 50 gels, to get information on five times as many genes, whereas running 50 gels with SAGE would reveal no statistically relevant information. Therefore, CLASS is a much more economic approach to identifying a gene expression pattern.

CLASS can be applied to any species, even those for which arrays are unavailable, and to mRNAs that have not yet been deposited on arrays. Thus, whereas use of targets generated by RAP-PCR on known arrays gives expression information on known genes, CLASS gives expression information on any gene, even if not previously encountered in libraries that have been arrayed. CLASS thus provides a low cost, relatively high throughput method for obtaining information on gene expression.

The invention also provides methods of measuring the level of nucleic acid molecules in a target using a statistically sampled target. Methods useful for generating a statistically sampled target have been previously described (WO 99/11823; McClelland et al., *supra*, 1997; Pesole et al., Biotechniques 25:112-123 (1998); Lopez-Nieto and Nigam, Nature Biotechnology 14:857-861 (1996)). An exemplary method for generating a statistically sampled target is statistically primed PCR (SP-PCR). The main difference between a statistical priming method and RAP-PCR is that the primers are selected by a computer program to determine the statistical occurrence of a nucleotide sequence in a group of nucleic acid molecules, rather than selecting primers arbitrarily.

A method for generating a statistically sampled target can be a directed statistical selection. For example, a program called GeneUP has been devised that uses an algorithm to select primer pairs to sample sequences in a list of interest, for example, a list of human mRNA associated with apoptosis, while excluding sequences in another list, for example, a list of abundantly expressed mRNA in human cells and structural RNAs such as rRNAs, Alu repeats and mtDNA (Pesole et al., *supra*, 1998). A directed statistical method provides a systematic determination of whether any given oligonucleotide matches any given nucleotide sequence and the number of different nucleic acid molecules to which a given oligonucleotide can bind. Such a directed statistical method can be used to generate a statistically sampled target useful in the invention.

Another method for generating a statistically sampled target is a Monte-Carlo statistical selection

method (Lopez-Nieto and Nigam, *supra*, 1996). A Monte-Carlo statistical selection method randomly pairs a set of primers using a Monte-Carlo method. A Monte-Carlo method approximates the solution of determining primers that can be used for amplification by simulating a random process of primer matching. A Monte-Carlo statistical method differs from a directed statistical method in that a directed statistical method provides a systematic determination of whether any given oligonucleotide matches any given nucleotide sequence and the number of different nucleic acid molecules to which a given oligonucleotide can bind.

In general, two arbitrarily sampled targets, generated using different pairs of arbitrary oligonucleotides, will hybridize to largely non-overlapping sets of nucleic acid molecules in a target sample. Similarly, two statistically sampled targets, generated using different pairs of statistical oligonucleotides, will hybridize to largely non-overlapping sets of nucleic acid molecules in a target. Generally, fewer than 100 products overlap among the most intensely hybridizing 2000 colonies in two differently primed reduced complexity target (see Example I). The pattern of expression is also almost entirely different from the pattern generated by directly labeling the whole mRNA population. However, as more nucleic acid molecules are sampled by additional arbitrary sampling of the RNA population or additional statistic sampling of the RNA population, the number of non-overlapping nucleic acid molecules sampled will decrease. To some extent, the efficiency of coverage of nucleic acid molecules can be improved by the use of statistically selected primers (Pesole et al., *supra*, 1998). Multiple arbitrarily

sampled targets generated by RAP-PCR could supply sufficient targets to cover all genes.

The methods described above for generating arbitrarily sampled targets and statistically sampled targets can be modified. For example, a subtraction strategy can be used to generate arbitrarily sampled targets or statistically sampled targets enriched for differentially regulated nucleic acids. A target from one source of nucleic acid molecules (A) is labeled, then mixed with a few-fold excess of unlabeled target from the other source (B). The whole mixture is denatured and added to the hybridization solution for binding to the probe. The amplified nucleic acid products present in both targets form double stranded nucleic acid molecules, and the remaining available labeled target is primarily from the differences between the two targets. The same experiment can be done with labeled target from source (B) and excess unlabeled target from source (A). The probe bound to both sets of subtracted targets are compared to detect differential gene expression. This procedure also partly quenches repeats present in the target cDNA mixtures. The use of such a subtraction method to generate an arbitrarily sampled target or statistically sampled target can thus be used to compare two conditions by using an unlabeled target from one condition to quench the labeled target from another condition.

A limitation of subtraction is that it can eliminate small differences in expression that can appear to be total absence of a mRNA. Furthermore, while subtraction is useful in a binary question, it is of limited utility in cases where a large number of conditions are to be compared, combinatorially.

Detection of specific binding is limited by background hybridization and incomplete blockage of repeats. Therefore, in addition to using the methods described above for generating reduced complexity targets, $Cot_1$ DNA can be used to quench nucleic acid repetitive elements. A $Cot_1$ DNA genomic fraction is enriched in repeats. A target that contains $Cot_1$ DNA is useful for looking at low abundance nucleic acid molecules that can be difficult to detect. Although low abundance sequences can be partly quenched by the use of total genomic DNA, $Cot_1$ DNA is useful for the more sophisticated arrays such as PCR-based arrays, where the signal to noise ratio is sufficiently high to be concerned about relatively poorly amplified products.

When generating an arbitrarily sampled target or a statistically sampled target, various promoters such as T7 polymerase, T3 polymerase, SP6 polymerase or others can be incorporated into a primer so that transcription with the corresponding polymerase is used to generate the target. Using transcription to generate the target has the advantage of generating a single stranded target. A primer comprising an RNA polymerase promoter can be used in combination with any other statistical or arbitrary primer.

An arbitrarily sampled target or a statistically sampled target can also be generated using digestion ligation. In this case, a population of nucleic acid molecules used to generate the target is digested with a restriction enzyme and an oligonucleotide primer is ligated to generate an amplified target. Ligation-mediated PCR is where a primer binding site or part of the primer binding site is placed on a template by ligation, for example, after site-specific cleavage.

Nested PCR can also be used to generate an arbitrarily sampled target or statistically sampled target. Nested PCR involves two PCR steps, with a first round of PCR performed using a first primer followed by PCR with a second primer that differs from the first primer in that it includes a sequence that extends one or more nucleotides beyond the first primer sequence.

Targets can be enriched for those that hybridize to a particular probe. Once a target generated by a particular arbitrary or statistically primed method has been used on a particular nucleic acid population and the resulting target used against a set of probes, then the set of targets that are detectably hybridized will be known. At that point it is possible to devise a new set of targets that includes only those that were detected or mostly those that were detected by that probe. For example, if a particular primer "A" is used for RAP-PCR using RNA from the human brain and the resulting target is hybridized to an array of cDNA clones, some of the clones will be detectably hybridized. It is then possible to make an array of only those probes that were hybridized by that particular target. Most of the cDNAs on the array can be expected to hybridize with a target developed from human brain RNA made with the same primer "A".

In some cases, the sequences of the nucleic acids that are the basis of targets are known. Some targets hybridize detectably with a particular probe and others do not. The sequence information associated with the targets can be used to deduce the rules of arbitrary or statistical priming events that resulted in the target that hybridized to those probes. Such information will help to predict what sequences are likely to be sampled

by a particular primer if that sequence occurs in the target. Such information can improve the estimates of which sequences are sampled efficiently and which sequences are sampled efficiently by a particular primer.

The methods of the invention are particularly useful for measuring the level of a molecule in a target using an array. As used herein, the term "array" or "array of molecules" refers to a plurality of molecules stably bound to a solid support. An array can comprise, for example, nucleic acid, oligonucleotide or polypeptide-nucleic acid molecules. It is understood that, as used herein, an array of molecules specifically excludes molecules that have been resolved electrophoretically prior to binding to a solid support and, as such, excludes Southern blots, Northern blots and Western blots of DNA, RNA and proteins, respectively.

As used herein, the term "non-dot blot" array refers to an array in which the molecules of the array are attached to the solid support by a means other than vacuum filtration or spotting onto a nitrocellulose or nylon membrane in a configuration of at least about 2 spots per $cm^2$.

As used herein, the term "peptide-nucleic acid" or "PNA" refers to a peptide and nucleic acid molecule covalently bound (Nielson, Current Opin. Biotechnol. 10:71-75 (1999)).

As used herein, the term "polypeptide," when used in reference to PNA, means a peptide, polypeptide or protein of two or more amino acids. The term is similarly intended to refer to derivatives, analogues and functional mimetics thereof. For example, derivatives

can include chemical modifications of the polypeptide such as alkylation, acylation, carbamylation, iodination, or any modification which derivatizes the polypeptide. Analogues can include modified amino acids, for example, hydroxyproline or carboxyglutamate, and can include amino acids that are not linked by peptide bonds. Mimetics encompass chemicals containing chemical moieties that mimic the function of the polypeptide regardless of the predicted three-dimensional structure of the compound. For example, if a polypeptide contains two charged chemical moieties in a functional domain, a mimetic places two charged chemical moieties in a spatial orientation and constrained structure so that the charged chemical function is maintained in three-dimensional space. Thus, all of these modifications are included within the term "polypeptide."

The solid support for the arrays can be nylon membranes, glass, derivatized glass, silicon or other substrates. The arrays can be flat surfaces such as membranes or can be spheres or beads, if desired. The molecules can be attached as "spots" on the solid support and generally can be spotted at a density of at least about $5/cm^2$ or $10/cm^2$, but generally does not exceed about $1000/cm^2$.

Various methods to manufacture arrays of DNA molecules have been described (reviewed in Ramsay, *supra*, 1998; Marshall and Hodgson, *supra*, 1998). Arrays are available containing nucleic acid molecules from various species, including yeast, mouse and human. The use of arrays is advantageous because differential expression of many genes can be determined in parallel.

One type of array contains thousands of PCR products per square centimeter. Arrays of PCR products from segments of mRNAs have been attached to glass, for example, and probed using cDNA populations from two sources. Each cDNA or cRNA population is labeled with a different fluorescent dye and hybridization is assessed using fluorescence (DeRisi et al., Nature Genet. 14:457-460 (1996); Schena et al., Science 270:467-470 (1995)). Arrays are also available containing over 5000 PCR products from selected I.M.A.G.E. clones. An array of PCR products also is available for every yeast ORF and for a subset of human ESTs.

Another type of array contains colonies of 18,432 *E. coli* clones, each carrying a different I.M.A.G.E. EST plasmid, and each spotted twice on a 22 x 22 cm membrane (Genome Systems). One advantage of using the arrays from the I.M.A.G.E. consortium is that more than 80% of the clones have single pass sequence reads from the 5' or 3' end, or both, deposited in the GenBank database. Thus, it is usually not necessary to clone or sequence any DNA to determine if there is a known gene or other ESTs that share the same sequence. UniGene clustering of human and mouse ESTs that appear to be from the same gene greatly aids in this process (http://www.ncbi.nlm.nih.gov/UniGene/index.html). Mapping onto chromosomes at a resolution of a few centiMorgans is also available for most of these clusters at the same web site. The clones on these arrays are all available to be used to probe nucleic acid molecules or to complete the sequencing (www-bio.llnl.gov). It is often possible to identify a close homolog in other species. In contrast to PCR product arrays and oligonucleotide arrays, which are free of other DNAs, each spotted EST is associated with *E. coli* genomic DNA

from the host. Thus, the clone arrays can have higher background than PCR arrays or oligonucleotide arrays.

If EST arrays are used, 5' RACE can be used to extend beyond the ESTs currently available (Zhang and Frohman, Methods Mol. Biol. 69:61-87 (1997)). When cDNA libraries that contain near full length clones are available and end sequenced, it will be possible to go from a differentially hybridized spot to a full length cDNA, directly.

Another class of arrays uses oligonucleotides that are either attached to a glass or silicon surface or manufactured by sequential photochemistry on the DNA chip (Chee et al., Science 274:610-614 (1996)). Such chips can contain tens of thousands of different oligonucleotide sequences per square centimeter. Arrays of oligonucleotide nucleic acid analogs such as peptide-nucleic acids, for example, can be prepared (Weiler et al., Nucleic Acids Res. 25:2792-2799 (1997)).

Hybridization of fingerprints to arrays has the huge advantage that there is generally no need to isolate, clone, and sequence the genes detected. In principle, all known human mRNAs will fit on three membranes (about 50,000 genes), or in a smaller area on glass arrays or other solid supports. At present, each fingerprint has a sufficient complexity to hybridize to over 2000 of the 50,000 known genes.

The use of arrays, which can have thousands of genes that can bind to a target, particular genes for further characterization can be selected based on desired criteria. For example, identified genes can be chosen that are already known and for which a new role in the

condition of interest can be deduced. Alternatively, some of the genes can be family members of known genes with known functions for which a plausible role can be determined.

In addition to arrays, a number of cDNA libraries are available, for example, from the I.M.A.G.E. consortium (www-bio.llnl.gov/bbrp/ image/image.html), including libraries available on nylon membranes, for example, from Research Genetics (Huntsville AL; www.resgen.com), Genome Systems (St. Louis MO; www.genomesystems.com), and the German Human Genome Project (www.rzpd.de). These libraries include clones from various human tissues, stages of development, disease states and other sources.

The methods of the invention include the step of detecting the amount of specific binding of the probe to the target. As disclosed herein, a variety of detection methods can be used. For example, if a detectable moiety is a radioactive moiety, the method of detection can be autoradiography or phosphoimaging. Phosphoimaging is advantageous for quantitation and shortened data collection time. If a detectable moiety is a fluorescent moiety, the method of detection can be fluorescence spectroscopy or confocal microscopy.

The methods of the invention use nucleic acid probes to measure the level of expression of a nucleic acid molecule in a target. If a radioactive moiety is attached to a target, for example, incorporation of the radioactive moiety can be by any enzymatic or chemical method that allows attachment of the radioactive moiety. For example, end-labeling can be used to attach a radioactive moiety to the end of a nucleic acid molecule.

Alternatively, a radioactive nucleotide, in particular a $^{32}P$-, $^{33}P$-, or $^{35}S$-labeled nucleotide, can be incorporated into the nucleic acid molecule during synthesis. The use of random primed synthesis is particularly useful for generating a high specific activity target. Generally, random primed synthesis generates approximately equal amounts of randomly primed nucleic acid molecules from both strands of double stranded PCR products, which will re-anneal to some degree during hybridization to the target (see Example I). If desired, the amount of re-annealing can be limited, for example, using exoIII digestion.

When generating a labeled target or probe, it is generally preferable to incorporate a labeled nucleotide that is not ATP or dATP. The use of labeled dATP can cause an increase in the background because any poly-A sequences in the target or probe will become heavily labeled and will hybridize to the strands containing poly-T stretches complementary to the poly-A tails present in all of the clones. Similarly, the use of dTTP would heavily label poly-T stretches complementary to the polyA tails in mRNA.

A fluorescent dye can also be attached to or incorporated in the probe or target. If desired, a different fluor detectable at different wavelengths can be incorporated into different targets and used simultaneously on the same probe. The use of different fluors is advantageous since multiple targets can be bound to the same probe and detected. A fluorescently labeled target can be detected using, for example, a fluorescent scanner or confocal microscope. Measuring the relative abundance of two targets simultaneously on the same array rather than on two different arrays

eliminates problems that arise due to differences in the hybridization conditions or the quantity of target PCR product on replicates of the same array. Nylon membranes are typically unsuitable for most commercially available fluorescent tags due to background fluorescence from the membrane itself.

Infrared dyes are also useful as detectable moieties for attachment to a probe or target. Infrared dyes are particularly useful with targets or probes such as arrays attached to nylon membranes, provided the membrane is free of protein.

When determining the level of a nucleic acid molecule in a target, some variation can occur, in particular for certain amplification products that are very sensitive to the amplification conditions. To control for variation in amplification products between nucleic acid targets, the target can be generated at two concentrations of nucleic acid molecules, differing by a factor of two or more. The use of various nucleic acid concentrations to generate a target to confirm differential expression is described herein (see Examples II and III).

The methods of the invention are directed to detecting specific binding of a target to a probe. When hybridizing a target to a probe, the specificity of binding is determined by the stringency of the hybridization conditions. The length of oligonucleotide primers and the temperature of the amplification reaction contributes to the final product. The products are a function of both the starting abundance of each target nucleic acid molecule and the quality of the match between the oligonucleotide primer and the amplified

nucleic acid target. For example, oligonucleotide primers of about 8 bases in length at reaction temperatures of about 60°C can be used to generate a target. Hybridization conditions can range, for example, from about 32°C in about 2x SSC to about 68° in about 0.1x SSC. The hybridization temperature can be, for example, about 40°C, about 45°C, about 50°C, about 55°C, about 60°C or about 65°C. Furthermore, the SSC concentration (see below) can be, for example, about 0.2x,0.3x, 0.5x, 1x or 1.5x.

The invention additionally provides a method for determining the relative amounts of nucleic acid molecules in two targets by comparing the amount of specific binding of a probe to the target, wherein the amount of specific binding corresponds to an expression level of the nucleic acid molecules in the target, to an expression level of the nucleic acid molecules in a second target. For example, if desired, the expression level in a first target, which can be a target for which the level of expression is unknown, can be compared to the expression level in a second target. The expression level in the second target can be determined, for example, by binding the same probe to the second target and determining the level of expression in the second target. The expression level in the first and second target can then be compared.

The relative expression level in a first target can also be compared to the expression level in a second target, where the abundance in the second target is already known. As used herein, the term "known" when used in reference to expression level of a nucleic acid molecule means that an abundance of a nucleic acid molecule has been previously determined. It is

understood that such a known abundance would apply to a particular set of conditions. It is also understood that, for the purpose of comparing the abundance of a nucleic acid molecule in an unknown target to a known abundance, the same method of measuring the abundance between the targets is used.

The invention also provides a method of identifying two or more differentially expressed nucleic acid molecules associated with a condition. The method includes the step of measuring the level of two or more nucleic acid molecules in a target, for example using an arbitrarily sampled target or a statistically sampled target, wherein the amount of specific binding of the target to the probe corresponds to an abundance of the nucleic acid molecules in the target. The method further includes the step of comparing the relative expression level of the nucleic acid molecules in the target to an expression level of the nucleic acid molecules in a second target, whereby a difference in expression level between the targets indicates a condition.

As used herein, the term "differentially expressed" means that the abundance of a molecule is expressed at different levels between two targets. Two targets can be from different cells or tissues, or the target can be from the same cell or tissue under different conditions. The condition can be, for example, associated with a disease state such as cancer, autoimmune disease, infection with a pathogen, including bacteria, virus, fungal, yeast, or single-celled and multi-celled parasites; associated with a treatment such as efficacy, resistance or toxicity associated with a treatment; or associated with a stimulus such as a

chemical, for example, a drug or a natural product, for example, a growth factor.

The methods of the invention are useful for determining differential gene expression between two targets. The methods of the invention can be applied to any system where differential gene expression is thought to be of significance, including drug and hormone responses, normal development, abnormal development, inheritance of a genotype, disease states such as cancer or autoimmunge disease, aging, infectious disease, pathology, drug treatment, hormone activity, aging, cell cycle, homeostatic mechanisms, and others, including combinations of the above conditions.

As disclosed herein, the abundance of nucleic acid molecules in two targets can be compared to identify two or more differentially expressed nucleic acid molecules (see Examples I to III). Using arbitrarily sampled targets, targets treated with and without EGF were hybridized with probes and a number of genes regulated by EGF were identified. EGF-regulated genes were found that increased in response to EGF and decreased in response to EGF (see Tables 1 and 2 in Examples II and III, respectively). The methods of the invention can therefore be used to determine nucleic acid molecules that increase in response to a stimulus or decrease in response to a stimulus (see Example II).

The arbitrarily sampled targets and statistically sampled targets used in the invention can readily detect less abundant nucleic acid molecules in a population. Therefore, the methods of the invention are particularly useful for identifying differentially

expressed nucleic acid molecules since differentially expressed nucleic acid molecules are often less abundant.

The methods of the invention can be applied to any two targets to determine differential gene expression. The methods of the invention can be used, for example, to diagnose a disease state. In such a case, a "normal" target is compared to a potential disease target to determine differential gene expression associated with the disease. A normal target can be a target sample of the same tissue nearby the diseased tissue from the patient. A normal target can also be a sample of the same tissue from a different individual. Using methods of the invention, a profile of normal expression can be established by determining a gene expression pattern in one to many normal target samples, which can then be used to compare to a potentially diseased target sample. Differential gene expression between the normal and diseased tissue can be used to diagnose or confirm a particular disease state. Furthermore, a collection of target samples obtained from known diseased tissue can similarly be determined to identify an abundance profile of the target reflecting gene expression associated with that disease. In such a case, comparison of a potential disease target sample to a known disease target sample with no differential gene expression would indicate that the potential disease target sample was associated with the disease.

The methods of the invention can also be used to assess treatment of an individual with a drug. The analysis of gene expression patterns associated with a particular drug treatment is also known as pharmacogenomics. The methods of the invention can be used to determine efficacy of a treatment, resistance to

a treatment or toxicity associated with a treatment. For example, a gene expression profile can be determined on an individual prior to treatment and after treatment for a particular disease or condition. A difference in gene expression can then be correlated with the effectiveness of the treatment. For example, if an individual is found to be responsive to treatment and if that treatment is associated with differential gene expression, the identification of differential gene expression can be used to correlate with efficacy of that treatment. As described above, a gene expression pattern associated with an untreated individual can be determined in the individual prior to treatment or can be determined in a number of individuals who have not been given the treatment. Similarly, a change in expression pattern associated with efficacy of the treatment can be determined in a number of individuals for which the treatment was efficacious. In such a case, comparison of a treated target sample to a known target sample associated with efficacious treatment with no differential gene expression would indicate that the treatment was likely to be efficacious. A similar approach can be used to determine the association of a treatment with toxicity of the treatment or resistance to a treatment. Resistance to a treatment could be associated with a change in expression pattern from an untreated target sample or could be associated with no change in the expression pattern compared to an untreated target sample.

The methods of the invention can also be used to determine co-regulated genes that can be potential targets for drug discovery. For example, a cell or organism can be treated with a stimulus and differential gene expression between the untreated target sample and

the target sample treated with a stimulus can be determined. The stimulus can be, for example, a drug or growth factor. A difference in the abundance of nucleic acid molecules between an untreated target sample and a target sample treated with a stimulus can be used to identify differential gene expression associated with the stimulus. Such a differential expression pattern can be used to determine if a target sample has been exposed to a stimulus. Additionally, the gene expression profile can be used to identify other chemicals that mimic the stimulus by screening for compounds that elicit the same gene expression profile as the original stimulus. Thus, the methods of the invention can be used to identify new drugs that have a similar effect as a known drug.

The methods of the invention are useful for identifying a marker for a pathway that correlates with a drug response by determining an abundance profile for a given target sample that reflects the expression profile of the source population of nucleic acids such as the source RNA. For example, the methods of the invention can be used to define the "neighborhood" of potential therapeutic targets by identifying several genes regulated in response to a drug, thereby providing "neighbors" in a pathway that are potential drug targets. The invention can also be used to define bad neighborhoods, for example, pathways that "failed" therapeutics, which can indicate that a particular pathway should not be perturbed. Additional insights into the function of a pathway can be obtained by sequencing any differentially expressed genes for which complete sequence information is unavailable. The methods are particularly useful for drug comparison. Correlation of gene expression patterns with a drug

response can be used to determine why two similar drugs have a somewhat different spectrum of effects.

With knowledge of the correlation between gene expression and response to a drug, drugs can be tested in cell types that are of more relevance to a particular disease or condition. By knowing the pathways that are present in a cell type associated with a pathology, predictions can be made regarding the drug responses of the cell type and thereby allow choice of drugs from a tested panels of drugs that are most likely to affect the pathology. The correlation of information on drug response and gene expression also can aid in choosing drugs that would be synergistic, for example, drugs that hit non-overlapping pathways, or, for example, drugs that affect overlapping pathways when genes in the overlap are targeted.

The methods of the invention can be applied to determining the response to a stimulus, in particular to determining a response to a stimulus for drug discovery. One potential application is to use the methods of the invention on the 60 cell lines in the National Cancer Institute (NCI) drug screening panel. These 60 cell lines are maintained by the NCI and used to assess drug activity.

For example, each of the 60 cell lines of the NCI panel can be used as a complex measuring device that reports the single variable of cell growth and, secondarily, apoptosis. Changes in each cell type's growth upon treatment with a chemical such as a drug is determined. Studies of tens of thousands of drugs, when compared over all 60 cell lines, have shown that similar effects on growth have proven to share mechanisms of

action. Comparing the response of the 60 cell lines to various drugs allows grouping of drugs according to their detailed chemical functionality. Consequently, the panel of cell lines has become one of the most important analytical tools for drug discovery.

The methods of the invention can be applied to analyzing drug response in the 60 cell lines of the NCI panel. As disclosed herein, the methods are applicable to determining differential gene expression, which can be correlated with the response of the cells to a particular drug. The methods can be used to identify many differentially expressed genes associated with a drug response. Therefore, an analysis of gene expression in untreated cells in the 60 cell line NCI drug screening panel can be used to determine a profile of gene expression, based on the presence or absence of mRNAs, that correlate with some of the many 10,000's of drugs that have been used on the panel.

Differential gene expression patterns are expected to correlate with drug response. Following identification of such a correlation in 30 of the cell lines, prediction of drug responses in the remaining 30 cell lines can be tested. This strategy circumvents the need to determine extensive expression profiles for all 60 cell lines for every new drug to find genes that correlate with the ability to respond to the drug. This strategy differs from previous methods in that differential expression of the gene after treatment does not need to occur. All that is necessary is that the gene be differentially regulated between cell types prior to treatment.

Each of the 60 cell lines has its characteristic response to drugs, and these responses depend on the cell's phenotype. The response of any cell to any drug depends on which genetic systems are operative in that cell. Once treated, the cell's genetic mechanisms are perturbed, leading to differential gene expression, differential protein modification, and a wide variety of other changes that can be subtle. Nonetheless, it is the ground state genetic pattern or profile of gene expression, before any exposure to drug, that determines how the cell responds to drugs.

The ground state of genetic profile is an important state to characterize for cells, for example, cells of the NCI panel. The ground state of the cell has predictive power for how a given cell will respond to a given drug. Furthermore, the ground state is the only unifying point of reference for the behavior of almost 100,000 different drugs and can be used to determine response to additional drugs.

For example, if two steroids and two alkylating agents are applied to the panel of 60 cell lines, and their growth spectra are compared, the average responses of the cell lines to the steroids tends to be similar, the average responses to the alkylating agents tend to be similar, but a comparison of responses to steroids versus alkylating agents show fewer similarities. This reflects the fact that steroids elicit their effects through naturally existing receptors, whereas alkylating agents elicit their effects by causing widespread damage. The signal transduction pathways for handling steroidal signals versus handling damage are largely different.

When a panel of steroids are used to challenge the 60 cell lines, some of the cells are growth accelerated, some growth inhibited, and some are indifferent to steroids. Much of this data is available on the NCI web site (http://www.nci.nih.gov/). An obvious next step is to examine gene responses to the steroids to see which genes are activated, which are inactivated, and which are indifferent. Each cell type's genes will respond differently, depending on which of about 30 steroid receptor genes are expressed in the cell type before steroid treatment.

The various responses of genes to steroids are cell type-dependent, in large part due to which receptors are present. By comparing the ground state gene expression of the NCI panel of cells, the spectrum of steroid receptor genes expressed in each cell type can be described, thereby explaining what is needed, in genetic terms, for a cell to be responsive to any particular steroid.

The drug-receptor, or hormone-receptor, relationship described above is one example of a correlation that can be drawn between the NCI panel baseline gene expression database and the NCI panel drug response database. Other drug responses can be readily determined. For example, drugs that induce apoptosis also induce gene expression, and different apoptotic responses correlating with cell type can be used to determine gene products that control apoptosis.

It is understood that methods of the invention can be applied to any cell type, in addition to the NCI panel of cells, for characterization of a response to a drug or other stimulus. The functional overlap between

drugs is an important concern in drug discovery. A study of the responses of genes to drugs in different cell types is useful because gene expression determines the response of the cell to the drug. The methods of the invention can therefore be applied to determine the response of one or more cell lines to a particular drug.

The methods can also be applied to characterize the ground state of the NCI panel of cells. The methods described herein can be used to correlate the response of tens of thousands of drugs with genes in the pathways regulated by the drug. The methods of the invention can be applied to determine an expression profile for the >80,000 drugs previously tested with the NCI panel of cells. The methods are applicable to determining coordinate mechanisms of drug action, likely pathways controlling drug activity, pathways that correlate with toxicity, apoptosis and other effects of drugs.

The invention also provides methods for the use of the patterns of gene expression by a panel of different untreated cells or tissues to correlate basal gene expression with susceptibility to a treatment, such as differences in the growth of cells, for example, the NCI panel of cells, in the presence of a drug, pathogen or other stimulus. The methods can be applied to determine genes and pathways that are present prior to treatment and also to correlate treatment with the phenotype induced by the treatment.

To obtain additional information on gene expression, the expression pattern of two different RNA populations from different conditions can be determined (McClelland et al., Nucleic Acids Res. 22:4419-4431 (1994); McClelland et al., Trends Genet. 11:242-246

(1995)). For example, if interested in apoptosis, using a target from a cell that has been stressed but which has not undergone apoptosis can be used to determine genes responsive to apoptosis, genes responsive to stress, and genes that respond to both. The identification of differentially regulated genes can be used to further characterize transcriptional activity of genes under various conditions. The genes can be further characterized to correlate promoters of regulated genes with signal transduction pathways that respond to a given condition.

When determining differential expression of a nucleic acid molecule, the determination that an RNA sampled in a target is differentially regulated is initially made by comparing differential abundance at two different concentrations of nucleic acid in the target sample. Abundance is determined for the nucleic acid molecules of the target sample for which no difference in abundance is observed at two different concentrations of RNA source. Only those hybridization events that indicate differential expression at both RNA concentrations in both RNA sources are used (see Examples II and III).

For hybridization to an array to determine differential expression, four membranes were used for radioactively labeled target, one for each of two concentrations of RNA for each of the two RNA samples compared (see Examples I to III). If two color fluorescence is used for detecting the target, then two membranes are used, one for each of the two concentrations of starting target sample nucleic acids, because the two targets with different detectable fluorescent markers can be mixed and applied to the same

probe. If a subsequent verification step is employed, for example, RT-PCR, one marker can be used for each target sample.

Confirmation of differential expression does not need a full length sequence and can be confirmed using RT-PCR of the known region. In particular, low stringency PCR can be used to generate products a few hundred bases in length (Mathieu-Daude et al., Mol. Biochem. Parasitol. 92:15-28 (1998)). This method generates internal "control" PCR products that can be used to confirm the quality of the PCR reaction and the quality and quantity of the RNA used.

The invention additionally provides a profile of five or more stimulus-regulated nucleic acid molecules. As used herein, the term "profile" refers to a group of two or more nucleic acid molecules that are characteristic of a target under a given set of conditions. The invention provides a profile comprising a portion of a nucleotide sequence selected from the group consisting of the nucleotide sequences referenced as SEQ ID NOS:1-45. The profile includes a portion of a nucleotide sequence of the GenBank accession numbers H11520, H11161, H11073, U35048, R48633, H28735, AF019386, H25513, H25514, M13918, H12999, H05639, L49207, H15184, H15124, X79781, H25195, H24377, M31627, H23972, H27350, AB000712, R75916, X85992, R73021, R73022, U66894, H10098, H10045, AF067817, R72714, X52541, H14529, M10277, H27389, D89092, D89678, H05545 , J03804, H27969, R73247, U51336, H21777, K00558, and D31765. The profile of the invention includes a portion of the nucleotide sequences encoding TSC-22, fibronectin receptor α-subunit, ray gene, X-box binding protein-1, CPE receptor, epithelium-restricted ets protein ESX and Vav-3.

The invention also provides a target comprising a portion of each of the nucleotide sequences referenced as SEQ ID NOS:1-45. The target includes a portion of a nucleotide sequence of the GenBank accession numbers H11520, H11161 H11073, U35048, R48633, H28735, AF019386, H25513, H25514, M13918, H12999, H05639, L49207, H15184, H15124, X79781, H25195, H24377, M31627, H23972, H27350, AB000712, R75916, X85992, R73021, R73022, U66894, H10098, H10045, AF067817, R72714, X52541, H14529, M10277, H27389, D89092, D89678, H05545 , J03804, H27969, R73247, U51336, H21777, K00558, and D31765. The invention also provides a probe comprising a portion of a nucleic acid sequence selected from the group consisting of SEQ ID NOS:1-45.

The invention further provides a substantially pure nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOS:1-45, or a functional fragment thereof, so long as the nucleic acid molecule does not include the exact SEQ ID NOS:1-45.

The invention additionally provides a method of measuring the amount of two or more nucleic acid molecules in a first target relative to a second target. The method includes the step of hybridizing a first amplified nucleic acid target comprising two or more nucleic acid molecules to a probe, wherein the target is amplified from a population of nucleic acid molecules using one or more oligonucleotides, wherein the oligonucleotide hybridizes by chance to a nucleic acid molecule in the population of nucleic acid molecules, wherein the amplification is not based on abundance of nucleic acids in the population of nucleic acid molecules, and wherein the amplified nucleic acids in the target are enhanced for less abundant nucleic acids in

the population of nucleic acid molecules. Further included in the method are the steps of detecting the amount of hybridization of the first amplified nucleic acid target to the probe, wherein the amount of hybridization corresponds to an abundance of the nucleic acid molecules in the first target; and comparing the abundance of the nucleic acid molecules in the first target to the abundance of the nucleic acid molecules in a second target, wherein the amplified nucleic acid target comprises a subset of nucleic acids in the initial nucleic acid populations.

The invention further provides a method of measuring the amount of two or more nucleic acid molecules in a first target relative to a second target. The method includes the step of hybridizing a first amplified nucleic acid target comprising 50 or more nucleic acid molecules to a probe, wherein the target is amplified from a population of nucleic acid molecules, wherein the amplification is not based on abundance of nucleic acids in the population of nucleic acid molecules, and wherein the amplified nucleic acids in the target are enhanced for less abundant nucleic acids in the population of nucleic acid molecules. The method further includes the steps of detecting the amount of hybridization of the amplified nucleic acid target to the probe, wherein the amount of hybridization corresponds to an expression level of the nucleic acid molecules in the first target; and comparing the abundance of the nucleic acid molecules in the first target to an abundance of the nucleic acid molecules in a second target, wherein the amplified nucleic acid target comprises a subset of nucleic acids in each nucleic acid population such as an RNA population.

As used herein, the term "hybridizes by chance," when referring to an oligonucleotide, means that hybridization of the oligonucleotide to a complementary sequence is based on the statistical frequency of the complementary sequence occurring in a given nucleic acid molecule. An oligonucleotide that hybridizes by chance is generated by determining the sequence of the oligonucleotide and subsequently determining if the oligonucleotide will hybridize to one or more nucleic acid molecules. The hybridization of such an oligonucleotide is not predetermined by the sequence of a known nucleic acid molecule and therefore occurs by chance. As such, an arbitrary oligonucleotide is considered to hybridize by chance since the oligonucleotides are determined without reference to the exact sequence to be amplified. In contrast, an oligonucleotide that does not hybridize by chance is one that is generated by first analyzing a known sequence and then identifying an exact sequence in the nucleic acid molecule that can be used as an oligonucleotide that will amplify an exact sequence between the oligonucleotides. The hybridization of such an oligonucleotide has been predetermined by the sequence of a known nucleic acid molecule and, therefore, does not occur by chance.

As used herein, the phrase "amplification is not based on abundance" means a target comprises nucleic acid molecules which are representative of the nucleic acid molecules in a population of nucleic acid molecules without regard to the relative amount of individual nucleic acid molecules in the population.

As used herein, the phrase "enhanced for less abundant nucleic acids" means that individual nucleic acid molecules that are less abundant in the population

of nucleic acid molecules are amplified so that the amount of these less abundant nucleic acid molecules would be increased relative to the amount of these nucleic acid molecules in the original population of nucleic acid molecules. Thus, the relative proportion of nucleic acid molecules in the population of nucleic acid molecules would not be maintained in the target.

As used herein, the term "single sample" when used in reference to a target means that the target is generated using nucleic acid molecules from a single cell, tissue or organism sample that has not been previously exposed to another sample. For example, if a target was generated from a population of nucleic acid molecules that was determined by the exposure of one sample to another, for example, the subtraction of the nucleic acid molecules of one sample from another, such a target would not be considered as coming from a single sample.

The following examples are intended to illustrate but not limit the present invention.

**EXAMPLE I**

Generation and Use of Arbitrarily Sampled Targets to Probe a DNA Array

This example describes the generation of an arbitrarily sampled target having reduced complexity to probe a DNA array to determine mRNA expression.

A DNA fingerprint was generated using RAP-PCR and was converted to high specific activity probe using random hexamer oligonucleotides (Genosys Biotechnologies; The Woodlands TX). Up to 10 μg of PCR product from

RAP-PCR was purified using a QIAQUICK PCR Purification Kit (Qiagen, Inc.; Chatsworth CA), which removes unincorporated bases, primers, and primer dimers smaller than 40 base pairs. The DNA was recovered in 100 µl of 10 mM Tris, pH 8.3. Random primed synthesis with incorporation of radioactive phosphorus from ($\alpha$-$^{32}$P)dCTP was used under standard conditions. 10% of the recovered fingerprint DNA (10 µl) was combined with 6 µg random hexamer oligonucleotide primer, and 1 µg of one of the fingerprint primers (Genosys) in a total volume of 28 µl, boiled for 3 min, then placed on ice. The hexamer/primer/DNA mix was mixed with 22 µl reaction mix to yield a 50 µl reaction containing a 0.05 mM concentration of three dNTP (dATP, dTTP and dGTP; minus dCTP), 100 µCi of 3000 Ci/mmol ($\alpha$-$^{32}$P) dCTP (10 µl), 1x Klenow fragment buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) and 8 U Klenow fragment (3.82 U/µl; Gibco-BRL Life Technologies; Gaithersburg MD). The reaction was performed at room temperature for 4 hr. For maximum target length, the reaction was chased by adding 1 µl of 2.5 mM dCTP and incubated for 15 min at room temperature followed by an additional 15 min incubation at 37°C. The unincorporated nucleotides and hexamers were removed with the Qiagen Nucleotide Removal Kit (Qiagen) and the purified products were eluted twice in 140 µl 10 mM Tris, pH 8.3.

For hybridization to the array, four membranes were used for radioactively labeled target, one for each of two concentrations of RNA for each of the two RNA samples to be compared. To prepare the cDNA filters (Genome Systems), the filters were prewashed in three changes of 2x SSC and 0.1% sodium dodecyl sulfate (SDS) in a horizontally shaking flat bottom container to reduce the residual bacterial debris. 20x SSC contains 3 M

NaCl, 0.3 M $Na_3$citrate-$2H_2O$, pH 7.0. The first wash was carried out in 500 ml for 10 min at room temperature. The second and third washes were carried out in 1 liter of prewarmed (50°C) prewash solution for 10 min each.

For prehybridization, the filters were transferred to roller bottles and prehybridized in 60 ml prewarmed (42°C) prehybridization solution containing 6x SSC, 5x Denhardt's reagent, 0.5% SDS, 100 µg/ml fragmented, denatured salmon sperm DNA (Pharmacia; Piscataway NJ) and 50% formamide (Aldrich; Milwaukee WI) for 1-2 hr at 42 °C. 50x Denhardt's solution contains 1% Ficoll, 1% polyvinylpyrrolidone and 1% bovine serum albumin, sterile filtered.

For hybridization, the prehybridization solution was removed and 7 ml prewarmed (42°C) hybridization solution, containing 6x SSC, 0.5% SDS, 100 µg/ml fragmented, denatured salmon sperm DNA and 50% formamide, was added. To decrease the background hybridization due to repeated sequences such as Alu repeats, long interspersed repetitive elements (LINE) or centromeric DNA repeats, sheared human genomic DNA (1 µg/ml stock concentration) was denatured in a boiling water bath for 10 min and immediately added to the hybridization solution to a final concentration of 10 µg/ml. Simultaneously, the labeled target (280 µl) was denatured in a boiling water bath for 4 min and immediately added to the hybridization solution. Hybridization was carried out at 42°C for 2 to 48 hrs, typically 18 hr, in a hybridization oven using roller bottles or sealed in a plastic bag and incubated in a water bath.

For the washes, the temperature was set to 55°C in the incubator oven (Techne HB-1D; VWR Scientific; San Francisco CA). The hybridization solution was poured off and the membrane was washed twice with 50 ml 2x SSC and 0.1% SDS for 5 min at room temperature. The membrane was then washed with 100 ml 0.1x SSC and 0.1% SDS and incubated for 10 min at room temperature. For the further washes, the wash solution, containing 0.1x SSC and 0.1% SDS, was prewarmed to 50°C and the filter was washed for 40 min in a roller bottle with 100 ml wash solution. The filter was then transferred to a horizontally shaking flat bottom container and washed in 1 liter of the wash solution for 20 min under gentle agitation. The filter was transferred back to a roller bottle containing 100 ml prewarmed 0.1x SSC and 0.1% SDS and incubated for 1 hr. The final wash solution was removed and the filter briefly rinsed in 2x SSC at room temperature.

After washing, the membranes were lightly dried with 3MM paper and the slightly moist membranes were wrapped in SARAN wrap. The membranes were exposed to X-ray film.

Figure 1 shows differential hybridization to clone arrays. All four images show a closeup of an autoradiogram for the same part of a larger membrane. Each image spans about 4000 double spotted *E. coli* colonies, each carrying a different EST clone. Panel A shows hybridization of 1 μg of polyA$^+$ RNA from confluent human keratinocytes that was radiolabeled during reverse transcription. About 500 clearly hybridizing clones can be seen. Panels B and C show RAP-PCR fingerprints with a pair of arbitrary primers that was performed on cDNA from oligo(dT) primed cDNA of confluent human keratinocytes

that were untreated (Panel B) or treated with EGF (Panel C). The pattern of hybridizing genes was almost identical in Panels B and C, but entirely different from that seen with total polyA+ RNA (compare to Panel A). The two radiolabeled colonies from one differentially expressed cDNA are indicated with an arrow. Differential expression of this gene was subsequently confirmed by specific RT-PCR (Trenkle et al., <u>Nucl. Acids Res.</u> 26:3883-3891 (1998)).

Figure 1D shows a RAP-PCR fingerprint with a different pair of arbitrary primers that was performed on RNA from confluent human keratinocytes. This pattern of hybridization is almost entirely different from that found with the previous primer pair (Panel B) and with mRNA (Panel A), with very few overlapping spots between Panel D and Panels A and B.

These results demonstrate that arbitrarily sampled targets, which have reduced complexity, allow detection of mRNAs that are not detectable using total message as a target. Thus, unlike a total message target, which detects mRNAs based on their abundance, an arbitrarily sampled target can be used to detect less abundant mRNAs.

**EXAMPLE II**

<u>An Arbitrarily Sampled Target Generated by RT-PCR Detects Genes Differentially Expressed in Response to EGF</u>

This example describes the use of RT-PCR with arbitrary primers to generate an arbitrarily sampled target for detecting differential gene expression upon treatment of cells with EGF.

An arbitrarily sampled target generated by RT-PCR was used to probe arrays for differential gene expression (Trenkle et al., Nucleic Acids Res. 26:3883-3891 (1998)). For RNA preparation, the immortal human keratinocyte cell line HaCaT (Boukamp et al., Genes Chromosomes Cancer 19:201-214 (1997)) was grown to confluence and maintained at confluence for two days. The media, DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin was changed one day prior to experiments. EGF (Gibco-BRL) was added at 20 ng/ml, or TGF-β (R&D Systems; Minneapolis MN) was added at 5 ng/ml. Treated and untreated cells were harvested after four hours by scraping the petri dishes in the presence of lysis buffer (RLT buffer; Qiagen) and homogenized through Qiashredder columns (Qiagen). On average, $7 \times 10^6$ cells, grown to confluency in a 100 mm diameter petri dish, yielded 40 μg of total RNA from the RNEASY total RNA purification kit (Qiagen). RNA, in 20 mM Tris, 10 mM $MgCl_2$ buffer, pH 8 was incubated with 0.08 U/μl of RNase free DNase and 0.32 U/μl of RNase inhibitor (both from Boehringer Mannheim Biochemicals; Indianapolis IN) for 40 min at 37°C and cleaned again using the RNEASY kit, which is important for removing small amounts of genomic DNA that can contribute to the fingerprints. RNA quantity was measured by spectrophotometry, and RNA samples were adjusted to 400 ng/μl in water. RNA samples were checked for quality and concentration by agarose gel electrophoresis and stored at -20°C.

For RNA fingerprinting, RAP-PCR was performed using standard protocols (McClelland et al., *supra*, 1994; Reverse transcription was performed on total RNA using four concentrations per sample (1000, 500, 250 and 125 ng per reaction) and a oligo d(T) primer (15-mer) (Genosys). RNA (5 μl) was mixed with 5 μl of buffer for a 10 μl

final reaction volume containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 0.2 mM of each dNTP, 0.5 µM of primer, and 20 U of MuLV-reverse transcriptase (Promega; Madison WI). RNA samples are checked for DNA contaminants by including a reverse transcriptase-free control in initial RAP-PCR experiments. The reaction was performed at 37°C for 1 hr, after a 5 min ramp from 25°C to 37°C. The enzyme was inactivated by heating the samples at 94°C for 5 min, and the newly synthesized cDNA was diluted 4-fold in water.

PCR was performed after the addition of a pair of two different 10- or 11-mer oligonucleotide primers of arbitrary sequence; pair A: GP14 (GTAGCCCAGC; SEQ ID NO:) plus GP16 (GCCACCCAGA; SEQ ID NO:), pair B: Nucl+ (ACGAAGAAGAAGAG; SEQ ID NO:) plus OPN24 (AGGGGCACCA; SEQ ID NO:). In general, there are no particular constraints on the primers except that they contain at least a few C or G bases, that the 3' ends are not complementary with themselves or the other primer in the reaction, to avoid primer dimers, and that primer sets are chosen that are different in sequence so that the same parts of mRNA are not amplified in different fingerprints.

Diluted cDNAs (10 µl) were mixed with the same volume of 2x PCR mixture containing 20 mM Tris, pH 8.3, 20 mM KCl, 6.25 mM $MgCl_2$, 0.35 mM of each dNTP, 2 µM of each oligonucleotide primer, 2 µCi α-($^{32}$P)-dCTP (ICN; Irvine CA) and 5 U AMPLITAQ DNA polymerase Stoffel fragment, (Perkin-Elmer-Cetus; Norwalk CT) for a 20 µl final reaction volume. Thermocycling was performed using 35 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 2 min.

A 3.5 µl aliquot of the amplification products was mixed with 9 µl of formamide dye solution, denatured at 85°C for 4 min, and chilled on ice. 2.4 µl was loaded onto a 5% polyacrylamide, 43% urea gel prepared with 1x TBE buffer containing 0.09 M Tris-borate, 0.002 M ethylene diamine tetraacetic acid (EDTA). The PCR products resulting from the four different concentrations of the same RNA template were loaded side by side on the gel.

Electrophoresis was performed at 1,700 V or at a constant power of 50-70 Watts until the xylene cyanol tracking dye reached the bottom of the gel (approximately 4 h). The gel was dried under vacuum and placed on Kodak BioMax X-Ray film for 16 to 48 hours.

For labeling of RAP-PCR products for use as targets to probe arrays, up to 10 µg of PCR product from RAP-PCR was purified using a QIAQUICK PCR Purification Kit (QIAGEN) which removes unincorporated bases, primers, and primer dimers under 40 base pairs. The DNA was recovered in 50 µl of 10 mM Tris, pH 8.3.

Random primed synthesis with incorporation of α-($^{32}$ P)-dCTP was performed essentially as described in Example I. Briefly, 10% of the recovered fingerprint DNA, typically about 100 ng in 5 µl, was combined with 3 µg random hexamer oligonucleotide primer and 0.3 µg of each of the fingerprint primers in a total volume of 14 µl, which was boiled for 3 min and then placed on ice.

The hexamer/primer/DNA mix was mixed with 11 µl reaction mix to yield a 25 µl reaction containing 0.05 mM of three dNTP (minus dCTP), 50 µCi of 3000 Ci/mmol α-($^{32}$P)-dCTP (5 µl), 1x Klenow fragment buffer, containing

50 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 8.0, and 4 U Klenow fragment (Gibco-BRL). The reaction was performed at room temperature for 4 hrs. For maximum target length, the reaction was chased by adding 1 µl of 1.25 mM dCTP and incubated for 15 min at 25°C, followed by an additional 15 min incubation at 37°C. The unincorporated nucleotides, hexamers and primers were removed with the Qiagen Nucleotide Removal Kit (Qiagen) and the purified products were eluted using two aliquots of 140 µl of 10 mM Tris, pH 8.3.

For labeling of poly(A)$^+$ mRNA and genomic DNA for use as a target, random hexamers were used to label poly(A)$^+$-selected mRNA and genomic DNA. Genomic DNA (150 ng) was labeled using the same protocol used for labeling the RAP-PCR products described above. Poly(A)$^+$ mRNA (1 µg) and 9 µg random hexamer in a volume of 27 µl were incubated at 70°C for 2 min and chilled on ice. The RNA/hexamer mix was mixed with 23 µl master mix, which contained 10 µl 5x AMV reaction buffer, containing 250 mM Tris-HCl, pH 8.5, 40 mM $MgCl_2$, 150 mM KCl, 5mM DTT, 1 µl three dNTP, each 33 mM (dATP, dTTP, dGTP; minus dCTP), 2 µl AMV reverse transcriptase (20 units; Boehringer Mannheim) and 10 µl 3000 Ci/mmol $\alpha$-($^{32}$P)-dCTP in a final volume of 50 µl. The reaction was incubated at room temperature for 15 min, ramped for 1 hour to 47°C, held at 47°C for 1 hr, and chased with 1 µl of 33 mM dCTP for another 30 min at 47°C. The labeled products were purified as described above.

For hybridization to the array, four membranes were used, one membrane for each of two concentrations of RNA for each of the two RNA samples to be compared. The cDNA filters (Genome Systems) were washed in three changes of 2x SSC and 0.1% SDS in a horizontally shaking

flat bottom container to reduce the residual bacterial debris. The first wash was carried out in 500 ml for 10 min at room temperature. The second and third washes were carried out in 1 liter of prewash solution, prewarmed to 55°C, for 10 min each wash.

For prehybridization, the filters were transferred to roller bottles and prehybridized in 60 ml prehybridization solution, prewarmed to 42°C, containing 6x SSC, 5x Denhardt's reagent, 0.5% SDS, 100 μg/ml fragmented, denatured salmon sperm DNA, and 50% formamide for 1-2 hrs at 42°C in a hybridization oven.

For hybridization, the prehybridization solution was removed and 7 ml hybridization solution, prewarmed to 42°C, containing 6x SSC, 0.5% SDS, 100 μg/ml fragmented, denatured salmon sperm DNA, and 50% formamide, was added. To decrease the background hybridization due to repeats such as Alu and Line elements, sheared human genomic DNA was denatured in a boiling water bath for 10 min and immediately added to the hybridization solution to a final concentration of 10 μg/ml. 10 ng/ml poly(dA) was added to block oligo d(T) stretches in the radiolabeled target. Simultaneously, the labeled target, in a total volume of 280 μl, was denatured in a boiling water bath for 4 min and immediately added to the hybridization solution. The hybridization was carried out at 42°C for 2-48 hrs, typically 18 hrs, in large roller bottles.

For the washes, the incubator oven temperature was set to 68°C. The hybridization solution was poured off and the membrane was washed twice with 50 ml 2x SSC and 0.1% SDS at room temperature for 5 min. The wash solution was then replaced with 100 ml 0.1x SSC and

0.1% SDS and incubated for 10 min at room temperature. For the further washes, the wash solution, containing 0.1x SSC and 0.1% SDS, was prewarmed to 68°C. The membranes were incubated 40 min in 100 ml of wash solution in the roller bottles, then the filters were transferred to horizontally shaking flat bottom containers and washed in 1 liter for 20 min under gentle agitation. The filters were transferred back to the roller bottles containing 100 ml 0.1x SSC and 0.1% SDS, prewarmed to 68°C, and incubated for 1 hr. The final wash solution was removed and the filters are briefly rinsed in 2x SSC at room temperature.

After washing, the membranes were blotted with 3MM paper, wrapped in SARAN wrap while moist, and exposed to X-ray film. The membranes were usually sufficiently radioactive that a one-day exposure with a screen revealed the top 1000 products on an array of 18,432 bacterial colonies carrying EST clones. Weaker targets or fainter hybridization events were visualized using an intensifying screen at -70°C for a few days.

For confirmation of differential expression, low stringency RT-PCR was used. The initial confirmation of differential expression was the use of two RNA concentrations per sample. Only those hybridization events that indicated differential expression at both RNA concentrations in both RNA samples were relied upon.

More than 70% of the I.M.A.G.E. consortium clones have single pass sequence reads from the 5' or 3' end, or both, deposited in the GenBank database. In cases where there is no prior sequence information available, the clones can be ordered from Genome Systems and sequenced. Sequences were used to derive PCR primers

of 18 to 25 bases in length using MacVector 6.0 (Oxford Molecular Group; Oxford UK). Generally, primers were chosen to generate PCR products of 50 to 250 base pairs and have melting temperatures of at least 60°C.

Reverse transcription was performed under the same conditions as in the RAP-PCR protocol described above, using an oligo-d(T) primer or a mixture of random 9-mer primers (Genosys). The PCR reaction was performed using the two pairs of specific primers described below (18 to 25-mers). The PCR conditions were the same as in the RAP-PCR fingerprint protocol except that 1.5 μM of each primer was used. A low stringency thermal profile was used: 94°C for 40 sec, 47°C for 40 sec, and 72°C for 1 min, for 19, 22 and 25 cycles in three separate reaction tubes. The reactions were carried out in three sets of tubes at different cycle numbers because the abundance of the transcripts, the performance of the primer pairs, and the amplifiability of the PCR products can vary. PCR products were run under the same conditions as above on a 5% polyacrylamide and 43% urea gel. The gel was dried and exposed to X-ray film for 18 to 72 hours. Invariance among the other arbitrary products in the fingerprint was used as an internal control to indicate the reliability of the relative quantitation.

Primer pairs (Genosys) were used for confirmation of differential expression.
For GenBank accession number H11520 (90 nucleotide product); primer A, AATGAGGGGGACAAATGGGAAGC (SEQ ID NO:); primer B, GGAGAGCCCTTCCTCAGACATGAAG (SEQ ID NO:).
For TSC-22 gene (GenBank accession numbers U35048, H11073, H11161; 179 nucleotide product); primer A, TGACAAAATGGTGACAGGTAGCTGG (SEQ ID NO:); primer B, AAGTCCACACCTCCTCAGACAGCC (SEQ ID NO).

For GenBank accession number R48633 (178 nucleotide product); primer A, CCCAGACACCCAAACAGCCGTG (SEQ ID NO); primer B, TGGAGCAGCCGTGTGTGCTG (SEQ ID NO:).

The array analyzed contains 18,432 *E. coli* colonies, each carrying a different I.M.A.G.E. consortium EST plasmid (www-bio.llnl.gov/bbrp/image/image.html), spotted twice on a 22x22 cm membrane (Genome Systems). The Genome Systems arrays are advantageous in that they contain by far the largest number of ESTs per unit cost. RNA fingerprinting for target preparation.

RAP-PCR amplifications were performed to look for differential gene expression in keratinocytes (HaCaT) when treated with EGF or TGF-β for four hours (Boukamp et al., *supra*, 1997). These experiments were designed to detect genes differentially regulated by EGF and TGF-β treatment in confluent keratinocytes. Using RAP-PCR, about 1% of the genes in normal or immortal keratinocytes responded to EGF, and fewer responded to TGF-β in this time frame.

Shown in Figure 2 are RAP-PCR fingerprints of RNA from confluent keratinocytes treated with TGF-β or EGF using multiple RNA concentrations and two sets of arbitrarily chosen primers. Reverse transcription was performed with an oligo-dT primer on 250, 125, 62.5 and 31.25 ng RNA in lanes 1, 2, 3, and 4, respectively. RNA was from untreated, TGF-β treated or EGF treated HaCaT cells, as indicated. RAP-PCR was performed with two sets of primers, GP14 and GP16 (Panel A) or Nuc1+ and OPN24 (Panel B). The sizes of the two differentially amplified RAP-PCR products are indicated with arrows (317 and 291 nucleotides).

In the first fingerprint shown in Figure 2A, two differentially regulated products were detected, which were cloned and sequenced. The sizes of these two products, 291 and 317 nucleotides, are indicated with arrows (see Figure 2A). The Genome Systems arrays used were chosen based on the presence of these two clones. This fingerprint was used to demonstrate that differentially regulated genes in an array can be identified without isolating, cloning and sequencing the RAP-PCR products. The fingerprint shown in Figure 2A and the second fingerprint shown in Figure 2B, which displayed no differential regulation in response to the treatments, were also used to demonstrate that fainter differentially regulated products not visible on the fingerprint gel could, nevertheless, be observed by the array approach.

The results obtained were highly reproducible. Using gel electrophoresis, there were no differences among the ~100 bands visible in any of the fingerprints from a single treatment condition performed at different RNA concentrations (see Figure 2). Similarly, more than 99% of the top 1000 clones hybridized by the targets derived from the fingerprint in Figure 2A were visible at both input RNA concentrations. Furthermore, more than 98% of the products were the same between the two treatment conditions, plus and minus EGF, at a single RNA concentration. These results indicated high reproducibility among the top 1000 PCR products in the RAP-PCR amplification.

The untreated control and EGF-treated samples were further characterized. RAP-PCR fingerprints shown in Figure 2 were converted into high specific activity radioactive targets by random primed synthesis using

α-($^{32}$P)-dCTP as described above. For each of the two conditions, EGF treated and untreated, fingerprints generated from RNA at two different concentrations were converted to target by random primed synthesis for each of the two different fingerprinting primer pairs. These radioactively labeled fingerprint targets were then used to probe by hybridizing to a set of identical arrays each containing 18,432 I.M.A.G.E. consortium cDNA clones. As controls, total genomic DNA and total poly(A)$^+$ mRNA were also labeled by random priming, as described above, and used as targets on identical arrays.

The RAP-PCR fingerprint targets, the total mRNA target and the genomic target were hybridized individually against replicates of a Genome Systems colony array. Genomic DNA was used as a blocking agent and as a competitor for highly repetitive sequences. Washing at 68°C in 0.1x SSC and 0.1% SDS removed virtually all hybridization to known Alu elements on the membrane, presumably because Alu elements are sufficiently diverged from each other at this wash stringency.

Shown in Figure 3 are autoradiograms from the same half of each membrane. All images presented are autoradiograms of the bottom half of duplicates of the same filter (Genome Systems) probed by hybridization with radiolabeled DNA. Panels A and B show hybridization of two RAP-PCR reactions generated using the same primers (GP14 and GP16) and derived from untreated (Panel A) or EGF treated (Panel B) HaCaT cells. Three double-spotted clones that show differential hybridization signals are marked on each array. The GenBank Accession numbers of the clone and the corresponding genes are H10045 and H10098, corresponding to *vav-3* and AF067817

(square) (Katzav et al., EMBO J. 8:2283-2290 (1989); H28735, gene unknown, similar to heparan sulfate 3-O-sulfotransferase-1, AF019386 (circle) (Shworak et al., J. Biol. Chem. 272:28008-28019 (1997); and R48633, gene unknown (diamond).

Figure 3 shows the results of hybridization of targets from these fingerprints to the arrays. As shown in Figure 3A and 3B, arrayed clones corresponding to the 291 nucleotide (*vav-3*, marked by square) and 317 nucleotide (similar to heparin sulfate N-sulfotransferase (N-HSST), marked by circle) RAP-PCR fragments are indicated. The sequences of these RAP-PCR fragments were determined. Also indicated on this array is a differentially regulated gene that could not be visualized on the original fingerprint gel (marked by diamond).

Comparing Figures 3A and 3B, a more than 10-fold down-regulation was observed for *vav-3* upon treatment with EGF. The gene corresponding to H28735 was up-regulated more than 10-fold with EGF treatment. The gene corresponding to R48633 was up-regulated about 3-fold with EGF treatment. These changes in gene expression in response to EGF were independently confirmed by RT-PCR.

These results indicate that RAP-PCR samples a population of mRNAs largely independently of message abundance. This is because the low abundance class of messages has much higher complexity than the abundant class, making it more likely that the arbitrary primers will find good matches. Unlike differential display, RAP-PCR demands two such arbitrary priming events, possibly biasing RAP-PCR toward the complex class.

Overall, these data suggest that the majority of the mRNA population in a cell (< 20,000 mRNAs) can be found in as few as ten RAP-PCR fingerprints. This result indicates that differential gene regulation can be detected by the combined fingerprinting and array approach even when the event cannot be detected using the standard gel electrophoresis approach.

Figure 3C shows an array hybridized with a RAP-PCR target using the same RNA as in panel A but with a different pair of primers, Nucl+ and OPN24. As shown in Figure 3C, using a different set of primers yields an entirely different pattern of hybridizing genes. Figure 3D shows an array hybridized with a cDNA generated by reverse transcription of 1 μg poly(A)$^+$-selected mRNA. Figure 3E shows an array hybridized with human genomic DNA labeled using random priming.

The data were analyzed in a number of ways. First, estimates were made of the overlap between the clones hybridized by each target. In all pairwise comparisons between all of the different types of targets, there was less than 5% overlap among the 500 clones that hybridized most intensely (compare Figure 3A, 3B, 3D, and 3E). Of the top 500 clones hybridized by the genomic target, which included nearly all clones known to contain the Alu repeats, less than 5% overlapped with the top 500 clones hybridized by the fingerprint targets or the total poly(A)$^+$ mRNA target. This indicated that, except for the case of a genomic target, there was no significant hybridization to dispersed repeats. The overlap among the clones hybridized by the two RAP-PCR fingerprints generated with different primers was less than 3%, and the overlaps of either fingerprint with the poly(A)$^+$ mRNA target were both less than 3%. Thus, most

of the cDNAs detected using a target from the fingerprints could not be detected using the total mRNA target. These results indicate that RAP-PCR samples a population of mRNAs largely independently of message abundance. This is because the low abundance class of messages has much higher complexity than the abundant class, making it more likely that the arbitrary primers will find good matches. Unlike differential display, RAP-PCR demands two such arbitrary priming events, possibly biasing RAP-PCR toward the complex class. Overall, these data suggest that the majority of the mRNA population in a cell (< 20,000 mRNAs) can be found in as few as ten RAP-PCR fingerprints.

A total of 30 differentially hybridizing cDNA clones were detected among about 2000 hybridizing colonies using targets derived from both sets of arbitrary primers (Figure 2) at a threshold of about three-fold differential hybridization. Twenty-two of these differentially hybridizing clones displayed differential hybridization at both RNA concentrations. These 22 were further characterized by RT-PCR. Differentially expressed genes exhibiting greater than a two-fold difference in expression in response to EGF treatment are shown in Table 1. For the results shown in Table 1, differential expression was confirmed by low stringency RT-PCR. The left column gives the accession numbers of the EST clones (5' or 3', or both when available). The right column gives the corresponding gene or the closest homolog. In cases of very low homologies, the gene is considered unknown. The cutoff for homology was p<e-20 in tblastx.

Table 1. Genes Regulated More than Two-fold After EGF Treatment of HaCaT Keratinocytes.

| Accession number | Gene name |
|---|---|
| **Up-regulated** | |
| H11520 (3') | unknown |
| H11161 (5')/H11073 (3') | TSC-22 (U35048) |
| R48633 (5') | unknown |
| H28735 (3') | similar to heparan sulfate 3-O-sulfotransferase-1 precursor (AF019386) |
| H25513 (5')/H25514 (3') | Fibronectin receptor α-subunit (M13918) |
| H12999 (5')/H05639 (3') | similar to Focal adhesion kinase (FAK2) (L49207) |
| H15184 (5')/H15124 (3') | ray gene (X79781) |
| H25195 (5')/H24377 (3') | X-box binding protein-1 (XBP-1) (M31627) |
| H23972 ('') | unknown |
| H27350 (5') | CPE-receptor (hCPE-R) (AB000712) |
| R75916 (5') | similar to semaphorin C (X85992) |
| **Down-regulated** | |
| R73021 (5')/R73022 (3') | epithelium-restricted Ets protein ESX (U66894) |
| H10098 (5')/H10045 (3') | vav-3 (AF067817) |

The eight false-positive clones that appeared to be regulated at only one concentration were further characterized. Of these eight, five false-positive clones showed differential hybridization at one concentration but were present and not regulated on the membranes for the other concentration. The most likely

source of this type of false-positive is the membranes. Although each clone is spotted twice, it is possible that occasionally one membrane received substantially more, or less, DNA in both spots than the other three membranes for these clones. However, this potential difference was easily detected and is rare, occurring only five times in over 2000 clones. The other three false-positive clones hybridized under only one treatment condition and at only one RNA concentration used for RAP-PCR. These three false-positive clones could be differentially expressed genes or could be false-positives from variable PCR products. However, the number of false positives was very low and were easily identified by comparing the results of two targets derived from PCR of different starting concentrations of RNA.

Differential expression was confirmed using low stringency RT-PCR. Only those hybridization events that indicated differential expression at both input RNA concentrations were further characterized. For confirmation of differential expression, RT-PCR was used with specific targets rather than Northern blots, which are much less sensitive than RT-PCR, because it was expected that many of the mRNAs would be rare and in low abundance. One of the advantages of using the arrays from the I.M.A.G.E. consortium is that more than 70% of the clones have single pass sequence reads from the 5' or 3' end, or both, deposited in the GenBank database.

Clones for which some sequence is available in the database were chosen for further characterization. Five of the 22 ESTs representing differentially regulated genes on the array had not been sequenced and two of the remaining 17 ESTs were from the same gene. The remaining 15 unique sequenced genes were aligned with other

sequences in the database in order to derive a higher quality sequence from multiple reads and longer sequence from overlapping clones. The UniGene database clusters human and mouse ESTs that appear to be from the same gene (Schuler, J. Mol. Med. 75:694-698 (1997)). This database greatly aids in the process of assembling a composite sequence from different clones of the same mRNA (http://www.ncbi.nlm.nih.gov/UniGene/index.html). These composite sequences were then used to choose primers for RT-PCR.

For each gene, two specific primers were used in RT-PCR under low stringency conditions similar to those used to generate RAP-PCR fingerprints. In addition to the product of interest, a pattern of arbitrary products was generated, which is largely invariant and behaves as an internal control for RNA quality and quantity, and for reverse transcription efficiency (Mathieu-Daude et al., *supra*, 1998). The number of PCR cycles was adjusted to between 14 to 25 cycles, according to the abundance of the product, in order to preserve the differences in starting template mRNA abundances. This is necessary because rehybridization of abundant products during the PCR inhibits their amplification, and the difference in product abundances diminishes as the number of PCR cycles increases (Mathieu-Daude et al., Nucleic Acids Res. 24:2080-2086 (1996)).

Low stringency RT-PCR experiments confirmed the differential expression of the two transcripts that were identified in the RAP-PCR fingerprints of Figure 2A and showed differential hybridization to the cDNA array (compare Figure 3A versus 3B). One of these differentially expressed genes corresponds to a new family member of the *vav* protooncogene family (Katzav et

al., *supra*, 1989; Katzav, Crit. Rev. Oncog. 6:87-97 (1995); Bustelo, Crit. Rev. Oncog. 7:65-88 (1996); Romero and Fischer, Cell Signal. 8:545-553 (1996)). The other differentially expressed gene has homology to heparan sulfate 3-O-sulfotransferase-1 (Shworak et al., *supra*, 1997).

The other 13 differentially expressed were also tested and 11 were confirmed using low stringency RT-PCR. Some of the differentially expressed genes are shown in Figure 4. Reverse transcription was performed at two RNA concentrations (500 ng, left column; 250 ng, right column). The reaction was diluted 4-fold in water and one fourth was used for low stringency RT-PCR at different cycle numbers. The RT-PCR products were resolved on polyacrylamide-urea gels. Shown are bands for the control (22 cycles); for GenBank accession number H11520 (22 cycles); for TSC-22, corresponding to GenBank accession numbers H11073 and H11161 (19 cycles) (Jay et al., Biochem. Biophys. Res. Commun. 222:821-826 (1996); Dmitrenko et al., Tsitol. Genet. 30:41-47 (1996); Ohta et al., Eur. J. Biochem. 242:460-466 (1996)); and for GenBank accession number R48633 (19 cycles). Genes corresponding to H11520 and TSC-22 are up-regulated about 8-10 fold with EGF treatment. The gene corresponding to R48633 is up-regulated about 3-fold with EGF treatment.

Of the two differentially expressed genes that were not confirmed, one proved unamplifiable. The other gene gave a product but appeared to not be differentially regulated when analyzed by RT-PCR.

RAP-PCR targets were very effective at detecting rare, low abundance mRNAs. Each fingerprint hybridized to a set of clones almost entirely different

from the set hybridized by a target derived from poly(A)$^+$-selected mRNA (see Figure 3). In addition, numerous other primer pairs, membranes, and sources of RNA consistently showed less than a 5% overlap between clones hybridized by any two fingerprints, or between a fingerprint and a total poly(A)$^+$-selected cDNA target. Detection of differentially expressed *vav-3* mRNA, which is a new member of the *vav* oncogene family, was attempted using a Northern blot of poly(A)$^+$-selected RNA. Despite being able to detect serially diluted vector down to the equivalent of a few copies per cell, *vav-3* mRNA was undetectable on the Northern blot, whereas RT-PCR confirmed expression. A G3PDH control was used to confirm that the conditions used in the Northern blot could detect a control gene. Therefore, *vav-3* appears to be a low abundance message that is represented in a RAP-PCR fingerprint as a prominent band.

The frequency of homologs of cDNAs detected by the RAP-PCR targets in the EST database was determined (>98% identity). This was compared to the frequency of homologs for a random set of other cDNAs on the same membrane. If the RAP-PCR fingerprints were heavily biased towards common mRNAs, then many would occur often in the EST database because it is partly derived from cDNA libraries that are not normalized or incompletely normalized. However, the cDNAs detected by RAP-PCR had frequencies in the EST database comparable to the frequencies for randomly selected cDNAs, including cases where the clone was unique in the database. These results indicate that sampling by arbitrarily sampled targets generated by RAP-PCR is at least as good as random sampling of the partly normalized libraries used to construct the array, and very different from that obtained for a target such as total mRNA target.

These results demonstrate that an arbitrarily sampled target generated using RT-PCR and arbitrary primers can detect genes differentially expressed in response to EGF.

## EXAMPLE III

### An Arbitrarily Sampled Target Generated by Differential Display Detects Genes Differentially Expressed in Response to EGF

This example shows the use of differential display to generate an arbitrarily sampled target and detection of differentially expressed genes responsive to EGF.

RNA was prepared from the human keratinocyte cell line HaCaT as described in Example II. Briefly, cells were grown to confluence and maintained at confluence for 2 days. The medium was changed 1 day prior to the experiment. EGF (Gibco-BRL) was added at 20 ng/ml. Treated and untreated cells were harvested after 4 hrs and total RNA was prepared with the RNEASY total RNA purification kit (Qiagen) according to the manufacturer's protocol. To remove remaining genomic DNA, the extracted total RNA was treated with RNase-free DNase (Boehringer Mannheim) and cleaned again using the RNEASY kit. The purified RNA was adjusted to 400 ng/μl in water and checked for quality by agarose gel electrophoresis.

For standard differential display, differential display was performed using the materials supplied in the RNAIMAGE kit (GenHunter Corporation; Nashville TN), AMPLITAQ DNA polymerase (Perkin-Elmer-ABI; Foster City CA) and $\alpha$-($^{32}$P)-dCTP according to the manufacturer's

protocol, except that each RNA template was used at four different concentrations, 800, 400, 200 and 100 ng per 20 µl reaction, with each anchored oligo(dT) primer (0.2 µM). The PCR reaction contained 2 µM dNTPs, for a total of 4 µM, including the carryover from the cDNA mix, 0.2 µM each primer, and one tenth of the newly synthesized cDNA, corresponding to 80, 40, 20 and 10 ng RNA. The anchored oligo(dT) primers were used in all possible combinations with four different arbitrary primers. The anchored oligo(dT) primers used were H-$T_{11}$G (HTTTTTTTTTTTG; SEQ ID NO:); H-$T_{11}$A (HTTTTTTTTTTTA; SEQ ID NO:); and H-$T_{11}$C (HTTTTTTTTTTTC; SEQ ID NO:), where H is AAGC, which is an arbitrary sequence used as a clamp to ensure the primers stay in register and have a high Tm at subsequent PCR steps. The arbitrary primers used were H-AP1 (AAGCTTGATTGCC; SEQ ID NO:); H-AP2 (AAGCTTCGACTGT; SEQ ID NO:); H-AP3 (AAGCTTTGGTCAG; SEQ ID NO:); and H-AP4 (AAGCTTCTCAACG; SEQ ID NO:).

For modified differential display, reverse transcription was performed using four different concentrations of each RNA template, 1000, 500, 250 and 125 ng per 10 µl reaction. The reaction mix contained 1.5 µM oligo(dT) anchored primers A$T_{15}$A, G$T_{15}$G, and $T_{13}$V, 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT, 0.2 mM each dNTP, 8 U RNase inhibitor (Boehringer Mannheim) and 20 U MuLV reverse transcriptase (Promega). The anchored primers were A$T_{15}$A (ATTTTTTTTTTTTTTTA; SEQ ID NO:); G$T_{15}$G (GTTTTTTTTTTTTTTTG; SEQ ID NO:); and $T_{13}$V (TTTTTTTTTTTTTV; SEQ ID NO:; where V is A, G or C)). The reaction mix was ramped for 5 min from 25°C to 37°C, held at 37°C for 1 hr, and finally the enzyme was inactivated at 94°C for 5 min. The newly synthesized cDNA was diluted 4-fold in water.

The PCR was performed after adding 10 µl of reaction mix to 10 µl of the diluted cDNAs, corresponding to 250, 125, 62.5 and 31.25 ng of RNA, to yield a 20 µl final reaction volume containing 2 µM anchored oligo(dT) primer, 0.4 µM arbitrary primer, either KA2 (GGTGCCTTTGG; SEQ ID NO:) or OPN28 (GCACCAGGGG; SEQ ID NO:), 2.5 units AMPLITAQ DNA polymerase Stoffel fragment (Perkin Elmer-ABI), 2 µCi α-($^{32}$P)-dCTP, 175 µM each dNTP, 10 mM Tris, pH 8.3, 10 mM KCl, and 3.125 mM $MgCl_2$. These concentrations do not include the carryover from the reverse transcription reaction. The reactions were thermocycled for 35 cycles of 94°C for 40 sec, 40°C for 1 min and 40 sec, and 72°C for 40 sec.

An aliquot of the PCR products resulting from the four different concentrations of the same RNA template were displayed side by side on a 5% polyacrylamide gel and visualized by autoradiography as described in Example II.

For labeling of differential display products for use as targets to probe arrays, random primed labeling of the differential display products was performed as described in Example II. The differential display PCR reactions (14 µl) were purified using a QIAQUICK PCR Purification Kit (Qiagen) and the DNA was recovered in 50 µl 10 mM Tris, pH 8.3. Random primed synthesis was performed using a standard protocol. Briefly, 5 µl of the recovered differential display products were combined with 3 µg random hexamers, boiled for 3 min and placed on ice. The hexamer/DNA mix was combined with the reaction mix to yield a 25 µl reaction containing 0.05 mM three dNTPs (minus dCTP), 50 µCi of 3000 Ci/mmol α-($^{32}$P)-dCTP, 1X Klenow fragment buffer, and 4 U Klenow fragment (Gibco-BRL). The reaction was

performed at room temperature for 4 hrs, chased for 15 min at room temperature by adding 1 µl of 1.25 mM dCTP, and incubated for an additional 15 min at 37°C. The unincorporated nucleotides and hexamers were removed with the Qiagen Nucleotide Removal Kit and the purified products were eluted using two aliquots of 140 µl 10 mM Tris, pH 8.3.

Hybridization to the array was performed essentially as described in Examples I and II. Briefly, the cDNA membranes (Genome Systems) were prewashed in three changes of prewash solution, containing 2x SSC and 0.1% SDS, in a horizontally shaking flat bottom container to reduce the residual bacterial debris. The first wash used 500 ml of prewash buffer for 10 min at room temperature. The second and third washes were each carried out in 1 liter of prewash solution, prewarmed to 55°C, for 10 min.

The membranes were transferred to large roller bottles and prehybridized in 60 ml prehybridization solution, prewarmed to 42°C, containing 6x SSC, 5x Denhardt's reagent, 0.5% SDS, 100 µg/ml fragmented, denatured salmon sperm DNA, and 50% formamide for 1-2 hrs at 42°C.

The prehybridization solution was removed, and 10 ml hybridization solution, prewarmed to 42°C and containing 6x SSC, 0.5% SDS, 100 µg/ml fragmented, denatured salmon sperm DNA and 50% formamide, was added to the bottles. To decrease the background hybridization due to repeats such as Alu and Line elements, sheared human genomic DNA was denatured in a boiling water bath for 10 min and immediately added to the hybridization solution to a final concentration of 10 µg/ml. An

aliquot of 10 ng/ml poly(dA) was added to block oligo (dT) stretches in the radiolabeled target. Simultaneously, the labeled target was denatured in a boiling water bath for 4 min and immediately added to the hybridization solution. The hybridizations were carried out at 42°C for 18-20 hrs.

Following hybridization, the hybridization solution was poured off and the membranes were thoroughly washed in six changes of wash solution, including a transfer of the membranes from the roller bottles to a horizontally shaking flat bottom container and back to the roller bottles, over 2-3 hrs. The stringency of the washes was increased stepwise from 2x SSC and 0.1% SDS at room temperature to 0.1x SSC and 0.1% SDS at 64°C. The separate washes were maintained at exactly the same indicated temperatures for all of the membranes. The last high stringency wash was at least 40 min to ensure exactly equilibrated temperatures in all bottles. The final wash solution was removed, and the membranes were briefly rinsed in 2x SSC at room temperature, blotted with 3MM paper, wrapped in SARAN wrap while moist, and placed against Kodak Biomax film (Eastman-Kodak; Rochester, NY).

Differential expression was confirmed using low stringency RT-PCR. The first level of confirmation was the use of two RNA concentrations per sample. Only those hybridization events that indicated differential expression at both RNA concentrations in both RNA samples were further characterized.

Nucleotide sequences, which were available from Genome Systems, the commercial source of the array, or were sequenced, were used to derive PCR primers of 18 to

25 bases in length using MacVector 6.0 (Oxford Molecular Group). Generally, primers were chosen that generate PCR products of 100 to 250 base pairs, have melting temperatures of at least 60°C, and were preferably located close to the polyadenylation site of the mRNA so as to reduce the chance of sampling family members.

Reverse transcription was performed on total RNA using two RNA concentrations per sample and an oligo-($dT_{15}$) primer (TTTTTTTTTTTTTTT; SEQ ID NO:; Genosys). The reactions contained 100 and 50 ng per liter total RNA, 0.5 µM oligo-($dT_{15}$) primer (SEQ ID NO:), 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 20 mM DTT, 0.2 mM of each dNTP, 0.8 U/µl RNase inhibitor (Boehringer Mannheim) and 2 U/µl of MuLV-reverse transcriptase (Promega). The reactions were ramped for 5 min from 25°C to 37°C and held at 37°C for 1 hr. The enzyme was inactivated by heating the reactions at 94°C for 5 min and the newly synthesized cDNA was diluted 4-fold in water.

Diluted cDNAs (10 µl) were mixed with 2x PCR mixture containing 20 mM Tris, pH 8.3, 20 mM KCl, 6.25 mM $MgCl_2$, 0.35 mM of each dNTP, 3 µM of each specific primer, 2 µCi α-($^{32}$P)-dCTP (ICN, Irvine, CA) and 2 U AMPLITAQ DNA polymerase Stoffel fragment (Perkin-Elmer-Cetus) for a 20 µl final reaction volume. A low stringency thermal profile was used: 94°C for 40 sec, 40°C for 40 sec, and 72°C for 1 min, for 17 and 19 cycles in separate tubes. The reaction was carried out in two sets of tubes at different cycle numbers because the abundance of the transcripts, the performance of the primer pairs and the amplifiability of the PCR products can vary. PCR products were run under the same conditions as described above on a 5% polyacrylamide and 43% urea gel. The gel

was dried and placed for 18 to 72 hours on a phosphoimager screen and read with a STORM phosphoimager (Molecular Dynamics; Sunnyvale CA). Invariance among the other arbitrary products in the fingerprint was used as an internal control to indicate the reliability of the relative quantitation. The gene-specific products from four sets of reactions per differentially regulated gene were quantitated using IMAGEQUANT Software (Molecular Dynamics).

Primer pairs were used to confirm differential expression.

For GenBank accession number R72714 (Egr-1)(155 nt product); primer A, CACGTCTTGGTGCCTTTTGTGTG (SEQ ID NO:); primer B, GAAGCTCAGCTCAGCCCTCTTCC (SEQ ID NO:).

For GenBank accession number H14529 (ACTB, β-actin)(174 nt product); primer A, CCAGGGAGACCAAAAGCCTTCATAC (SEQ ID NO:); primer B, CACAGGGGAGGTGATAGCATTGC (SEQ ID NO:).

For GenBank accession number H27389 (A+U-rich element RNA binding factor)(144 nt product); primer A, GTGCTTTTCAAAGATGCTGCTAGTG (SEQ ID NO:); primer B, GCTCAATCCACCCACAAAAACC (SEQ ID NO:).

For GenBank accession number H05545 (protein phosphatase 2A catalytic subunit)(141 nt product); primer A, TCCTCTCACTGCCTTGGTGGATG (SEQ ID NO:); primer B, CACAGCAAGTCACACATTGGACCC (SEQ ID NO:).

For GenBank accession number H27969 (103 nt product); primer A, CCAAAGACATTCAGAGGCATGG (SEQ ID NO:); primer B, GAGGTGGGGAAGGATACAGCAG (SEQ ID NO:).

For GenBank accession number R73247 (inositol tris phosphate kinase)(168 nt product); primer A, GAAAAGGGTTGGGGAGAAGCCTC (SEQ ID NO:); primer B, TCTCTAGCGTCCTCCATCTCACTGG (SEQ ID NO:).

For GenBank accession number H21777 (α-tubulin isoform 1)(155 nt product); primer A, ACAACTGCATCCTCACCACCCAC (SEQ

ID NO:); primer B, GGACACAATCTGGCTAATAAGGCGG (SEQ ID NO:).

Total RNA was obtained from immortalized HaCaT keratinocytes, treated and untreated with EGF, as described in Example II (Boukamp et al., *supra*, 1997). The first differential display protocol tried was the RNAimage kit 1 (cut G50'; GenHunter. The anchor primers, oligo(dT)-G (H-$T_{11}$G; SEQ ID NO:), oligo(dT)-C (H-$T_{11}$C; SEQ ID NO:) or oligo(dT)-A (H-$T_{11}$A; SEQ ID NO:), were used for reverse transcription, and then each cDNA was used for PCR in combination with four different arbitrary primers, H-AP1 (SEQ ID NO:), H-AP2 (SEQ ID NO:), H-AP3 (SEQ ID NO:) and H-AP4 (SEQ ID NO:).

As shown in Figure 5, the fingerprints were resolved on a denaturing acrylamide gel to determine the quality of the reactions. Differential display reactions were performed using the RNAIMAGE kit protocol (GenHunter Corporation) according to the manufacturer's suggestion except that four different starting concentrations of 800, 400, 200 and 100 ng of total RNA were used. One tenth of this material was then used for PCR. The anchored oligo(dT) primer H-$T_{11}$C (SEQ ID NO:) was used with two different arbitrary primers, H-AP3 (SEQ ID NO:) and H-AP4 (SEQ ID NO:), as indicated. The arbitrary primer H-AP4 (SEQ ID NO:) was used with two different anchored oligo(dT) primers, H-$T_{11}$C (SEQ ID NO:) and H-$T_{11}$A (SEQ ID NO:). The reactions that share either the arbitrary primer or the anchored oligo(dT) primer showed almost no visible overlap in the visible bands.

Figure 5B shows differential display using a different set of primers. Differential display was performed using the arbitrary primer KA2 (SEQ ID NO:)

with three different anchored oligo(dT) primers, $T_{13}V$ (SEQ ID NO:), $AT_{15}A$ (SEQ ID NO:), and $GT_{15}G$ (SEQ ID NO:), as indicated. The differential display protocol was adjusted to yield more mass and a higher complexity of the generated products. The starting concentrations of RNA were 1000, 500, 250 and 125 ng. One fourth of this material was then used for PCR. As observed in Figure 5A, using different oligo(dT) anchored primers changes the pattern of the displayed bands almost entirely.

The fingerprints generated about 30 to 50 clearly visible products (see Figure 5A). Fingerprints were generally reproducible in the range from 100 to 800 ng of total mRNA used in these experiments, with very few RNA concentration dependent products. Three of the most reproducible fingerprints that shared either a oligo(dT) anchored primer or an arbitrary primer (Figure 5A) were radiolabeled by random priming in the presence of three unlabeled dNTPs and $\alpha$-($^{32}$P)-dCTP, and each was used to probe identical arrays of 18,000 double spotted *E. coli* colonies carrying ESTs from the I.M.A.G.E. consortium. The arrays were hybridized and washed as described above.

The kit protocol used 0.2 µM of the arbitrary primer and 4 µM dNTPs compared to 1 µM primers and 200 µM dNTPs used in the RAP-PCR protocol described in Example II. The fingerprint reaction contained less than 40 ng of product in 20 µl, presumably because of limiting components. This was about five times less DNA than used in the method described in Example II. For this reason, it took about ten days with an intensifying screen in order to obtain an adequate exposure of X-ray film. Approximately 500 products were easily discernible with each target after a sufficient exposure. The number of

reliably observable genes is usually increased by at least two-fold or more when using a phosphoimager screen, indicating the greater sensitivity of phosphoimaging compared to X-ray film. Furthermore, pooling of separate labeled fingerprints into the same target can increase throughput even further.

In order to reduce the exposure time for target hybridization to arrays, experiments were performed at the higher concentration of primer and dNTPs described in Example II using RAP-PCR protocols (Figure 5B). These experiments yielded the expected increase in product mass and a corresponding reduction in exposure times for arrays.

The selectivity of oligo(dT) primers was determined using different anchor bases. As shown in Figure 6, differential display reactions were hybridized to cDNA arrays. The differential display products generated as described in Figure 5A, with the primers $GT_{15}G$ (SEQ ID NO:) and KA2 (SEQ ID NO:) from untreated (Figure 6A) and EGF treated (Figure 6B) HaCaT cells, were labeled by random priming and hybridized to cDNA arrays. A section representing less than 5% of a membrane is shown with a differentially regulated gene indicated by an arrow. Figure 6C shows hybridization of differential display products generated with the primers $AT_{15}A$ (SEQ ID NO:) and KA2 (SEQ ID NO:) from untreated HaCaT cells. Comparing Figure 6A versus 6C, there is a significant overlap of hybridization signals that were not obvious from the polyacrylamide display (compare to Figure 5B, lanes $AT_{15}A$/KA2 versus $GT_{15}G$/KA2).

When the arbitrary primer was changed while keeping the same anchor primer, the pattern of clones

hybridized changed almost entirely, with typically less than 5% overlap between any two fingerprints. In contrast, targets containing the same arbitrary primer and different anchored primers shared about 30% of the clones to which they hybridized. Figure 6A and 6C show examples of such shared products from a small portion of an array.

Similar observations were made using fingerprints generated under a wide variety of conditions, including the protocols and primers from the GenHunter kit, modified protocols, and protocols using primers independent of those in the GenHunter kit. The possibility of this overlap being due to repeats was excluded by the use of genomic and total mRNA targets against the same membranes.

The overlap among targets that had different anchored primers but shared the same arbitrary primer was not reflected in any noticeable similarity in the fingerprint products when resolved on a denaturing polyacrylamide gel. For example, the targets used in Figure 6A and 6C are shown in Figure 5B and show no easily discerned similarities, despite having 30% of the products in common. Many of the shared products were among the most intensely hybridizing clones on the array. Therefore, some of the products visible on the gel could share the arbitrary primer at one end but, during PCR, the products are preferentially primed at multiple different locations in the opposite direction by the different anchored primers. This would result in fingerprints that had little or no similarity in a polyacrylamide display while being compatible with the observation that targets with the same arbitrary primer

but different anchored primers overlap by 30% in the clones to which they hybridize.

Shared products are a general phenomenon for anchored fingerprints that share an arbitrary primer under a fairly wide range of conditions. Overlap among fingerprints can be avoided by not using the same arbitrary primer with different anchored primers.

Comparison of the pattern of hybridizing clones with that generated by total genomic DNA indicated that the clones hybridizing to a target generated by the GenHunter fingerprint did not generally contain the Alu repetitive element that occurs in a few percent of mRNA 3' untranslated regions (UTRs). The clones hybridized by the target did not overlap significantly with clones hybridized by a total cDNA target derived from reverse transcription of poly(A)$^+$ mRNA, indicating that the genes sampled were not heavily biased towards the most abundant RNAs. These results are consistent with results obtained using only arbitrary primers for fingerprinting (see Example II) and indicate that arbitrary priming combined with anchored oligo(dT) priming can be used to monitor rare genes in cDNA arrays. These results also confirm that RAP-PCR and differential display are not heavily biased toward abundant transcripts.

Among over 2000 clones surveyed for differential gene expression between untreated and EGF treated HaCaT cells, there were 29 different clones that appeared to clearly reflect differential expression at one RNA concentration. The 12 clones having the highest signal to noise ratio and differential expression ratio were chosen and specific primers were designed for RT-PCR. An example of one of these differentially

expressed genes is indicated by an arrow in Figure 6A versus 6B.

Differential expression of at least 1.5-fold was confirmed for seven genes, which are shown in Figure 7. Reverse transcription was performed at twofold different RNA concentrations. The reactions were diluted 4 fold in water and low stringency PCR was performed at different cycle numbers. The amount of input RNA/cDNA for each PCR reaction was 125 ng, left column and 250 ng, right column. The reactions shown in Figure 7 were carried out for 10 cycles and resolved on polyacrylamide-urea gels. Shown are products for the control (unregulated) and genes differing by at least 1.6-fold. The regulated genes shown correspond to GenBank accession numbers R72714, H14529, H27389, H05545, H27969, R73247, and H21777.

The regulation of the genes shown in Figure 7 are summarized in Table 2. Identified genes regulated by four hr treatment with EGF, corresponding GenBank accession numbers, and the fold-increase in expression relative to untreated cells are shown.

Table 2. EGF Regulated Genes.

| Gene | Accession # | Fold Up-regulation by EGF |
|---|---|---|
| EGR1 | R72714, X52541 | 8.3±3.4 |
| ACTB, beta-actin | H14529, M10277 | 2.0±0.3 |
| A+U-rich element RNA binding factor | H27389, D89092, D89678 | 1.9±0.3 |
| Protein phosphatase 2A catalytic subunit | H05545, J03804 | 1.6±0.4 |
| Unknown | D31765, H27969 | 1.6±0.4 |
| Inositol tris phosphate kinase | R73247, U51336 | 1.6±0.3 |
| Alpha-tubulin isoform 1 | H21777, K00558 | 1.6±0.3 |

Egr-1 was previously known to be differentially regulated by EGF in other cell types (Iwami et al., Am. J. Physiol. 270:H2100-H2107 (1996); Kujubu et al., J. Neurosci. Res. 36:58-65 (1993); Cao et al., J. Biol. Chem. 267:1345-1349 (1992); Ito et al., Oncogene 5:1755-1760 (1990)). The observations of changes in β-actin and α-tubulin expression are likely associated with the dramatic change in morphology these cells undergo after EGF treatment. Regulation of β-actin and α-tubulin genes by EGF has been observed in other cell types (Torok et al., J. Cell Physiol. 167:422-433 (1996); Hazan and Norton, J. Biol. Chem. 273:9078-9084 (1998); Shinji et al., Hepatogastroenterology 44:239-244 (1997); Ball et al., Cell Motil. Cytoskeleton 23:265-278 (1992)). These observations independently validate the treatments and the method used to detect differential expression.

The regulation of protein phosphatase 2A mRNA has not previously been observed but is consistent with the role of this protein in transduction of the EGF signal (Chajry et al., Eur. J. Biochem. 235:97-102 (1996)). Similarly, the gene associated with the metabolism of inositol phosphates had not previously been shown to be regulated by EGF but such regulation is consistent with the previous observation of increases in the compounds generated by this enzyme after EGF treatment in another ectodermal cell type (Contreras, J. Neurochem. 61:1035-1042 (1993)). Regulation of two other genes by EGF, an unknown gene, with GenBank accession number H27969, and an RNA binding protein, with GenBank accession number D89692, was not previously reported in any cell type. GenBank accesssion number D31765 corresponds to KIAA0061.

Five other genes were not confirmed to be regulated when RT-PCR was used. The number of false positives can vary from experiment to experiment and depends on the quality of the fingerprints and on the quality of the commercially available membranes. The number of false positives can be limited by using two RNA concentrations on arrays before confirmation by RT-PCR, as described in Example II. These experiments involved only a single concentration because the primary purpose was to determine the efficiency of coverage and overlap among targets made by the oligo(dT)-X anchored priming method. Nevertheless, over half of the differentially hybridizing clones observed at one concentration correspond to differentially expressed genes. When two array hybridizations were performed for each treatment at two different input template concentrations, the error rate was well below 10%.

These results demonstrate that an arbitrarily sampled target generated using differential display and arbitrary primers can detect genes differentially expressed in response to EGF.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.

We claim:

1. A method of measuring the level of two or more nucleic acid molecules in a target, comprising:

(a) contacting a probe with a target comprising two or more nucleic acid molecules, wherein said nucleic acid molecules are arbitrarily sampled and wherein said arbitrarily sampled nucleic acid molecules comprise a subset of the nucleic acid molecules in a population of nucleic acid molecules; and

(b) detecting the amount of specific binding of said target to said probe.

2. The method of claim 1, wherein said target comprises one or more less abundant nucleic acid molecules of said population.

3. The method of claim 1, wherein said less abundant nucleic acid molecule is less than 10% as abundant as the most abundant nucleic acid molecule in said population.

4. The method of claim 1, wherein said less abundant nucleic acid molecule is less than 1% as abundant as the most abundant nucleic acid molecule in said population.

5. The method of claim 1, wherein said less abundant nucleic acid molecule is less than 0.1% as abundant as the most abundant nucleic acid molecule in said population.

6. The method of claim 1, wherein said less abundant nucleic acid molecule is less than 0.01% as abundant as the most abundant nucleic acid molecule in said population.

7. The method of claim 1, wherein said target is generated using one or more arbitrary oligonucleotides.

8. The method of claim 1, wherein said target is generated using RNA arbitrarily primed polymerase chain reaction (RAP-PCR).

9. The method of claim 1, wherein said target is generated using differential display.

10. The method of claim 1, wherein said target is generated using digestion-ligation.

11. The method of claim 1, wherein said target is generated using a primer comprising an RNA polymerase promoter and an RNA polymerase.

12. The method of claim 11, wherein said RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 polymerase.

13. The method of claim 1, wherein said target is amplified.

14. The method of claim 13, wherein said amplified target is generated using polymerase chain reaction.

15. The method of claim 1, wherein said target is not amplified.

16. The method of claim 1, wherein said probe is an array of molecules.

17. The method of claim 16, wherein said molecules on said array are nucleic acid molecules.

18. The method of claim 16, wherein said molecules on said array are oligonucleotides.

19. The method of claim 16, wherein said molecules on said array are polypeptides.

20. The method of claim 16, wherein said molecules on said array are peptide-nucleic acids.

21. The method of claim 1, wherein said target comprises 10 or more nucleic acid molecules.

22. The method of claim 1, wherein said target comprises 20 or more nucleic acid molecules.

23. The method of claim 1, wherein said target comprises 50 or more nucleic acid molecules.

24. The method of claim 1, wherein said target comprises 100 or more nucleic acid molecules.

25. The method of claim 1, wherein said target comprises 1000 or more nucleic acid molecules.

26. The method of claim 1, further comprising comparing said amount of specific binding of said target

to said probe, wherein said amount of specific binding corresponds to an expression level of said nucleic acid molecules in said target, to an expression level of said nucleic acid molecules in a second target.

27. The method of claim 26, wherein said expression level of said nucleic acid molecules in said second target is known.

28. The method of claim 26, wherein said expression level of said nucleic acid molecules in said second target is determined by contacting said second target with said probe and detecting the amount of specific binding of said probe to said second target.

29. A method of measuring the level of two or more nucleic acid molecules in a target, comprising:

(a) contacting a probe with a target comprising two or more nucleic acid molecules, wherein said nucleic acid molecules are statistically sampled and wherein said statistically sampled nucleic acid molecules comprise a subset of the nucleic acid molecules in a population of nucleic acid molecules; and

(b) detecting the amount of specific binding of said target to said probe.

30. The method of claim 29, wherein said target comprises one or more less abundant sequences of said population.

31. The method of claim 30, wherein said less abundant sequence is less than 10% as abundant as the most abundant sequence in said population.

32. The method of claim 30, wherein said less abundant sequence is less than 1% as abundant as the most abundant sequence in said population.

33. The method of claim 30, wherein said less abundant sequence is less than 0.1% as abundant as the most abundant sequence in said population.

34. The method of claim 30, wherein said less abundant sequence is less than 0.01% as abundant as the most abundant sequence in said population.

35. The method of claim 29, wherein said statistically sampled target is enhanced for complexity of unrelated nucleic acid molecules.

36. The method of claim 29, wherein said target is generated using one or more statistical oligonucleotides.

37. The method of claim 36, wherein said statistical oligonucleotides are selected based on rank of complexity binding.

38. The method of claim 36, wherein said statistical oligonucleotides are enhanced for complexity binding.

39. The method of claim 29, wherein said target is generated using directed statistical selection.

40. The method of claim 29, wherein said target is generated using Monte-Carlo statistical selection.

41. The method of claim 29, wherein said target is generated using digestion-ligation.

42. The method of claim 29, wherein said target is generated using a primer comprising an RNA polymerase promoter and an RNA polymerase.

43. The method of claim 42, wherein said RNA polymerase is selected from the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 polymerase.

44. The method of claim 29, wherein said target is amplified.

45. The method of claim 44, wherein said amplified target is generated using polymerase chain reaction.

46. The method of claim 29, wherein said target is not amplified.

47. The method of claim 29, wherein said probe is an array of molecules.

48. The method of claim 47, wherein said molecules on said array are nucleic acid molecules.

49. The method of claim 47, wherein said molecules on said array are oligonucleotides.

50. The method of claim 47, wherein said molecules on said array are polypeptides.

51. The method of claim 47, wherein said molecules on said array are peptide-nucleic acids.

52. The method of claim 29, wherein said nucleic acid target comprises 10 or more nucleic acid molecules.

53. The method of claim 29, wherein said nucleic acid target comprises 20 or more nucleic acid molecules.

54. The method of claim 29, wherein said nucleic acid target comprises 50 or more nucleic acid molecules.

55. The method of claim 29, wherein said nucleic acid target comprises 100 or more nucleic acid molecules.

56. The method of claim 29, wherein said nucleic acid target comprises 1000 or more nucleic acid molecules.

57. The method of claim 29, further comprising comparing said amount of specific binding of said target to said probe, wherein said amount of specific binding corresponds to an abundance of said nucleic acid molecules in said target, to an abundance of said nucleic acid molecules in a second target.

58. The method of claim 57, wherein said abundance of said nucleic acid molecules in said second target is known.

59. The method of claim 57, wherein said abundance of said nucleic acid molecules in said second target is determined by contacting said second target

with said probe and detecting the amount of specific binding of said probe to said second target.

60. A method of identifying two or more differentially expressed nucleic acid molecules associated with a condition, comprising:

(a) measuring the level of two or more nucleic acid molecules in a target according to the method of claim 1, wherein said amount of specific binding of said target to said probe corresponds to an expression level of said nucleic acid molecules in said target;

(b) comparing said expression level of said nucleic acid molecules in said target to an expression level of said nucleic acid molecules in a second target, whereby a difference in expression level between said targets indicates a condition.

61. The method of claim 60, wherein said condition is associated with a disease state.

62. The method of claim 60, wherein said disease state is selected from the group consisting of cancer, autoimmune disease, infectious disease, aging, developmental disorder, proliferative disorder, neurological disorder.

63. The method of claim 60, wherein said condition is associated with a treatment.

64. The method of claim 63, wherein said difference in expression level indicates an efficacy of said treatment.

65. The method of claim 63, wherein said difference in expression level indicates a resistance to said treatment.

66. The method of claim 63, wherein said difference in expression level indicates a toxicity of said treatment.

67. The method of claim 60, wherein said condition is associated with a stimulus.

68. The method of claim 67, wherein said stimulus is a chemical.

69. The method of claim 68, wherein said chemical is a drug.

70. The method of claim 67, wherein said stimulus is a growth factor.

71. The method of claim 67, wherein said growth factor is epidermal growth factor (EGF).

72. The method of claim 71, wherein said target comprises a portion of a nucleic acid sequence selected from the group consisting of nucleic acids referenced as SEQ ID NOS:1-45.

73. The method of claim 67, wherein said stimulus is radiation.

74. The method of claim 67, wherein said stimulus is stress.

75. The method of claim 60, wherein said target is derived from skin cells.

76. The method of claim 75, wherein said skin cells comprise keratinocytes.

77. The method of claim 60, wherein said target is derived from a tumor.

78. The method of claim 67, wherein said stimulus is a pathogen.

79. A profile comprising five or more stimulus-regulated nucleic acid molecules.

80. The profile of claim 79, wherein said profile comprises ten or more stimulus-regulated nucleic acid molecules.

81. The profile of claim 79, wherein said profile comprises 100 or more stimulus-regulated nucleic acid molecules.

82. The profile of claim 79, wherein said profile comprises 1000 or more stimulus-regulated nucleic acid molecules.

83. The profile of claim 80, wherein said stimulus is epidermal growth factor.

84. The profile of claim 83, comprising a portion of a nucleotide sequence selected from the group consisting of the nucleotide sequences referenced as SEQ ID NOS:1-45.

85. A profile obtained by the method of claim 1.

86. The profile of claim 85, wherein said profile comprises two or more nucleic acid molecules.

87. The profile of claim 85, wherein said profile comprises 5 or more nucleic acid molecules.

88. The profile of claim 85, wherein said profile comprises 10 or more nucleic acid molecules.

89. The profile of claim 85, wherein said profile comprises 100 or more nucleic acid molecules.

90. A profile obtained by the method of claim 29.

91. The profile of claim 90, wherein said profile comprises two or more nucleic acid molecules.

92. The profile of claim 90, wherein said profile comprises 5 or more nucleic acid molecules.

93. The profile of claim 90, wherein said profile comprises 10 or more nucleic acid molecules.

94. The profile of claim 90, wherein said profile comprises 100 or more nucleic acid molecules.

95. A target comprising a portion of each of the nucleotide sequences referenced as SEQ ID NOS:1-45.

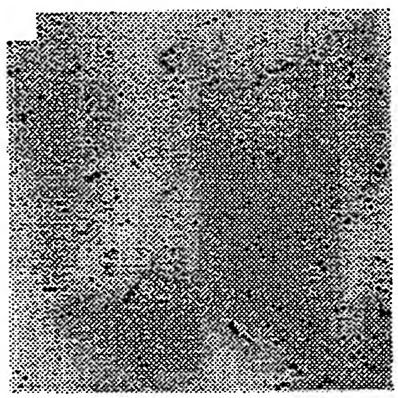


FIG. 1A

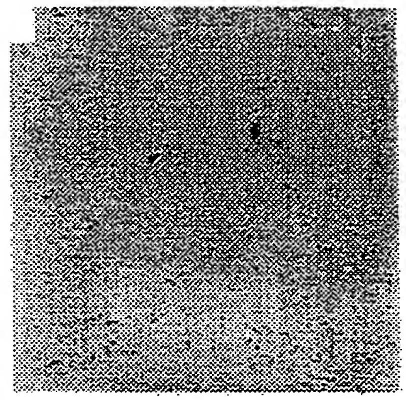


FIG. 1B

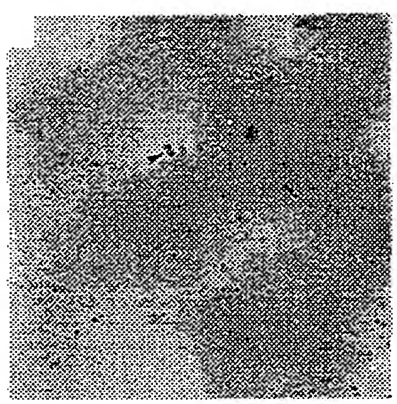


FIG. 1C

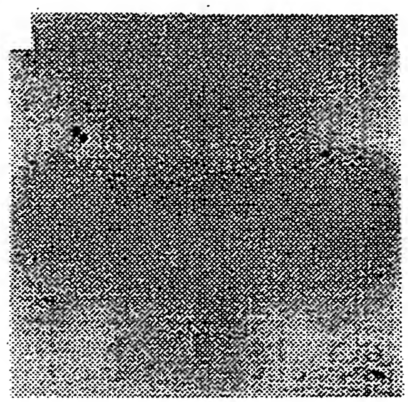
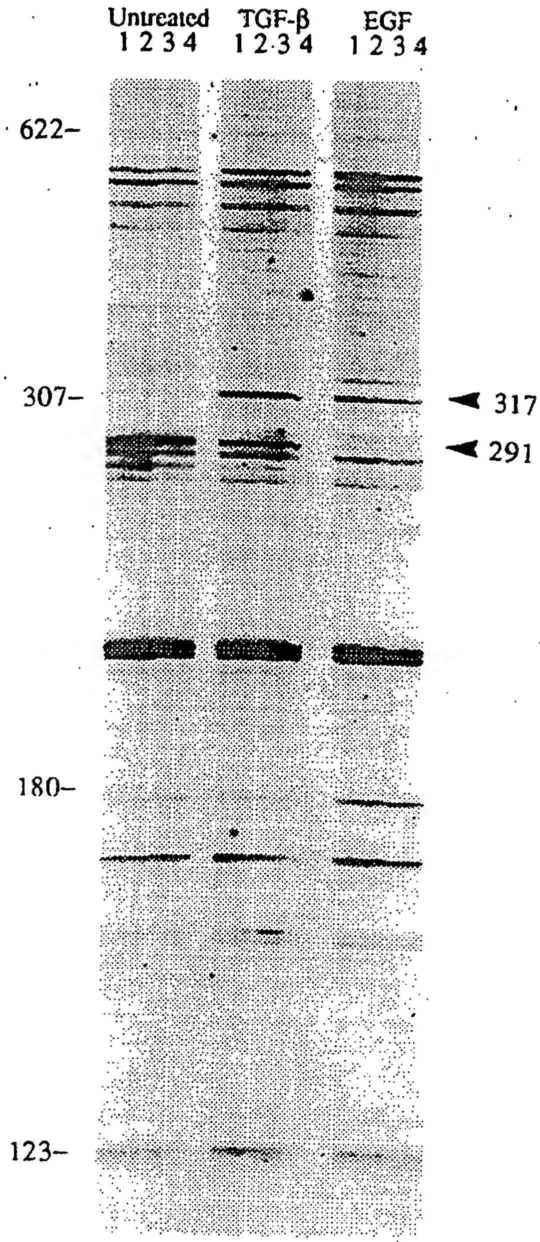


FIG. 1D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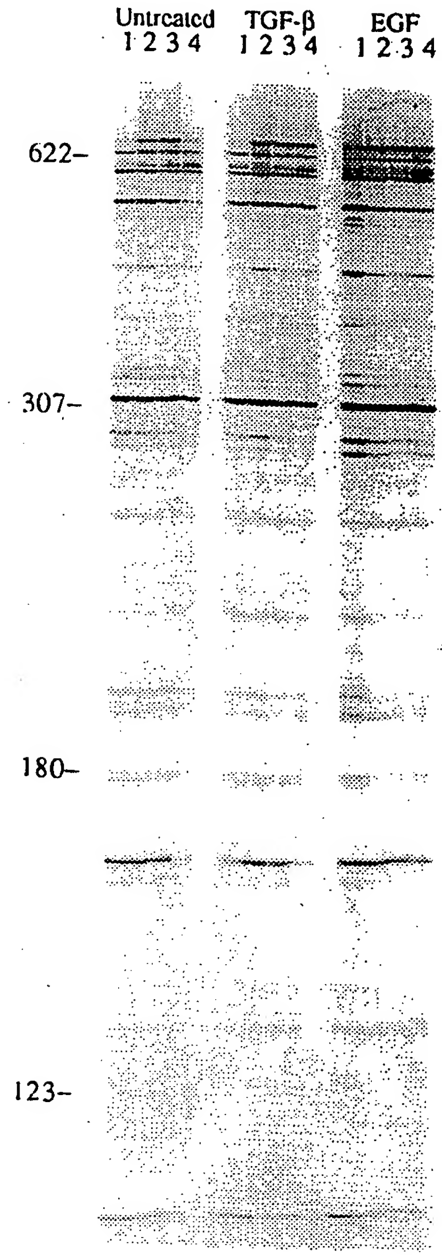
1 2 3 4
1 2 3 4
1 2 3 4
622–
307–
180–
123–
317
291

FIG. 2A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TGF-β
EGF
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1 2 3 4
1 2 3 4
622–
307–
180–
123–

FIG. 2B

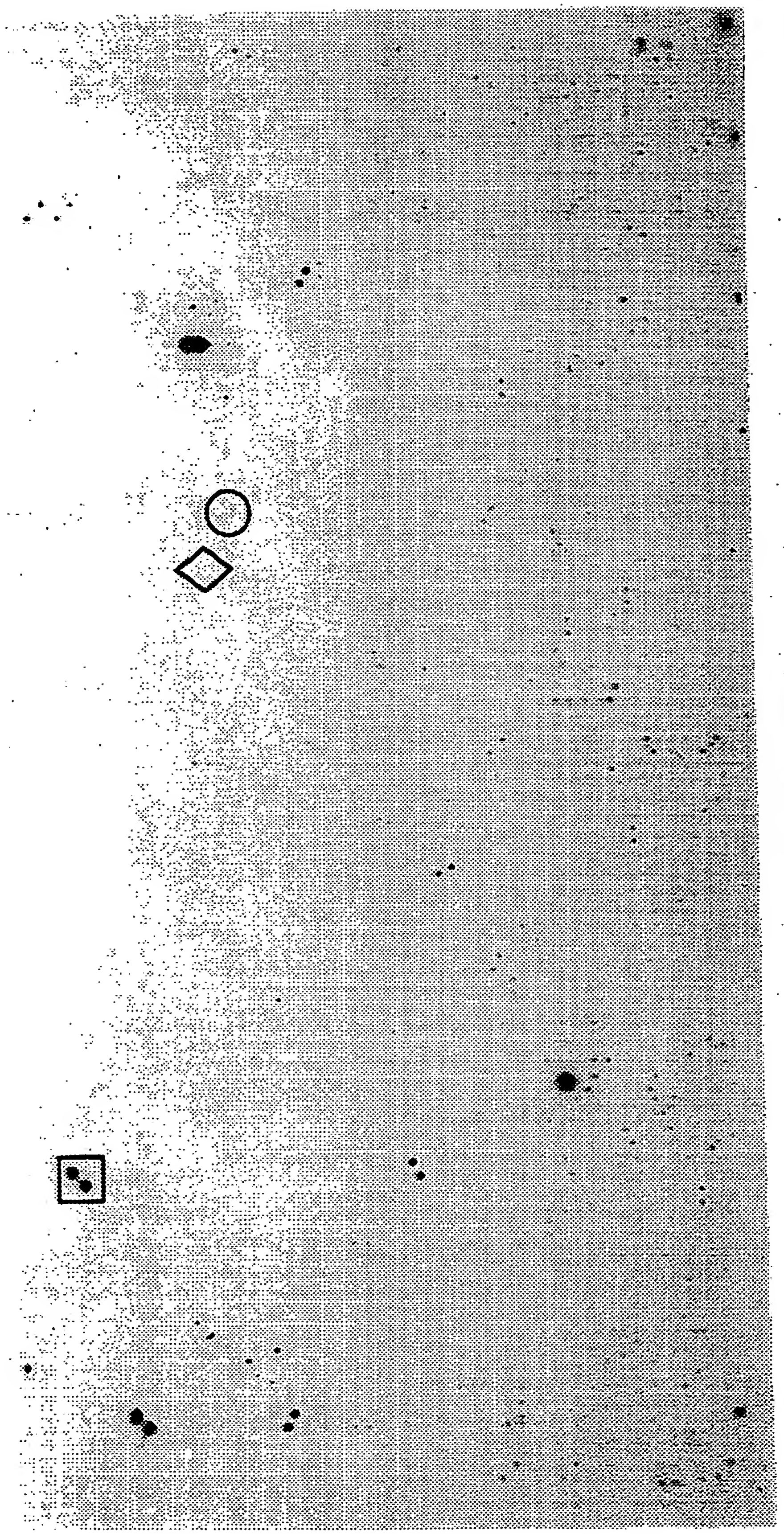


FIG. 3A

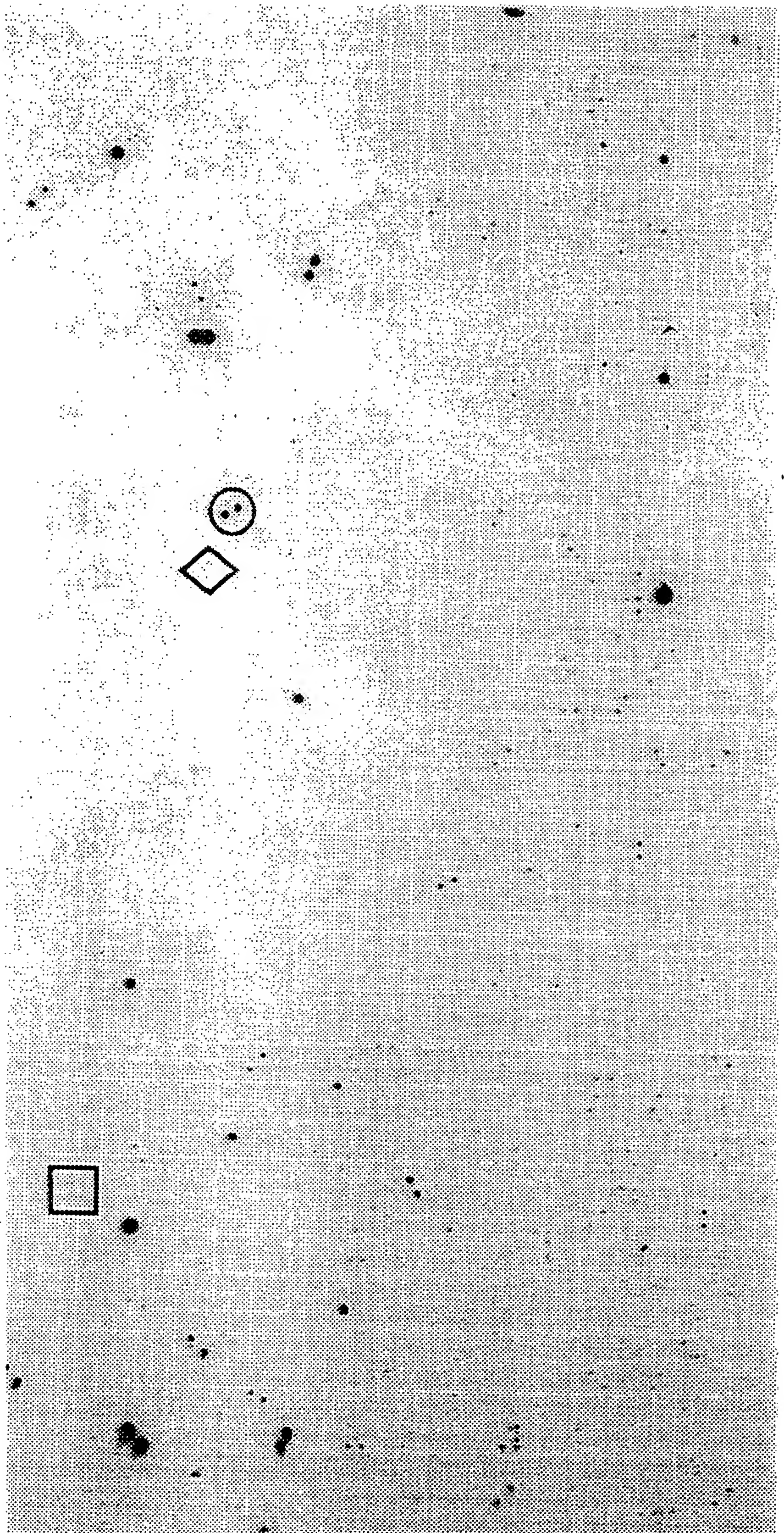


FIG. 3B

FIG. 3C

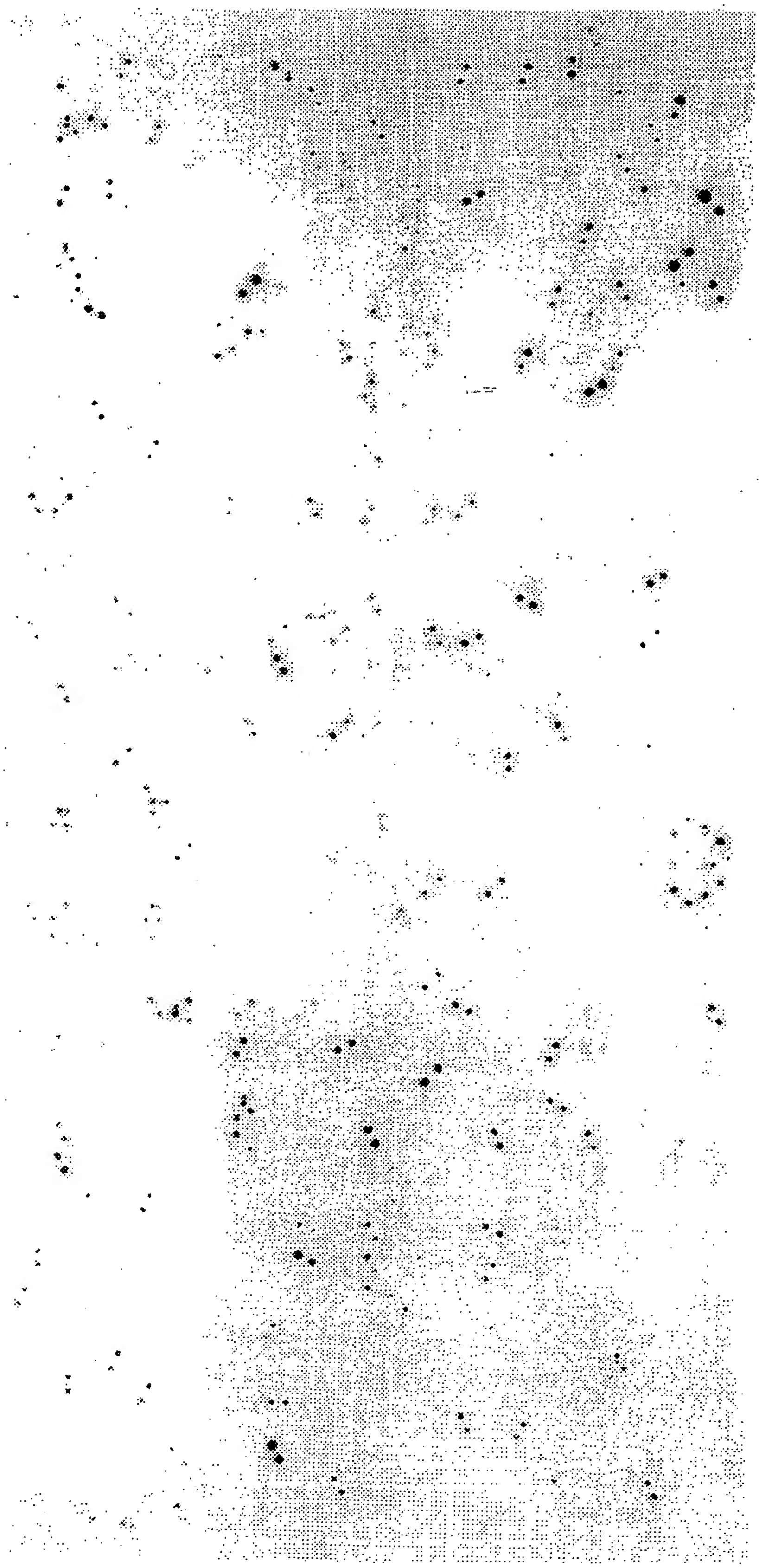


FIG. 3D

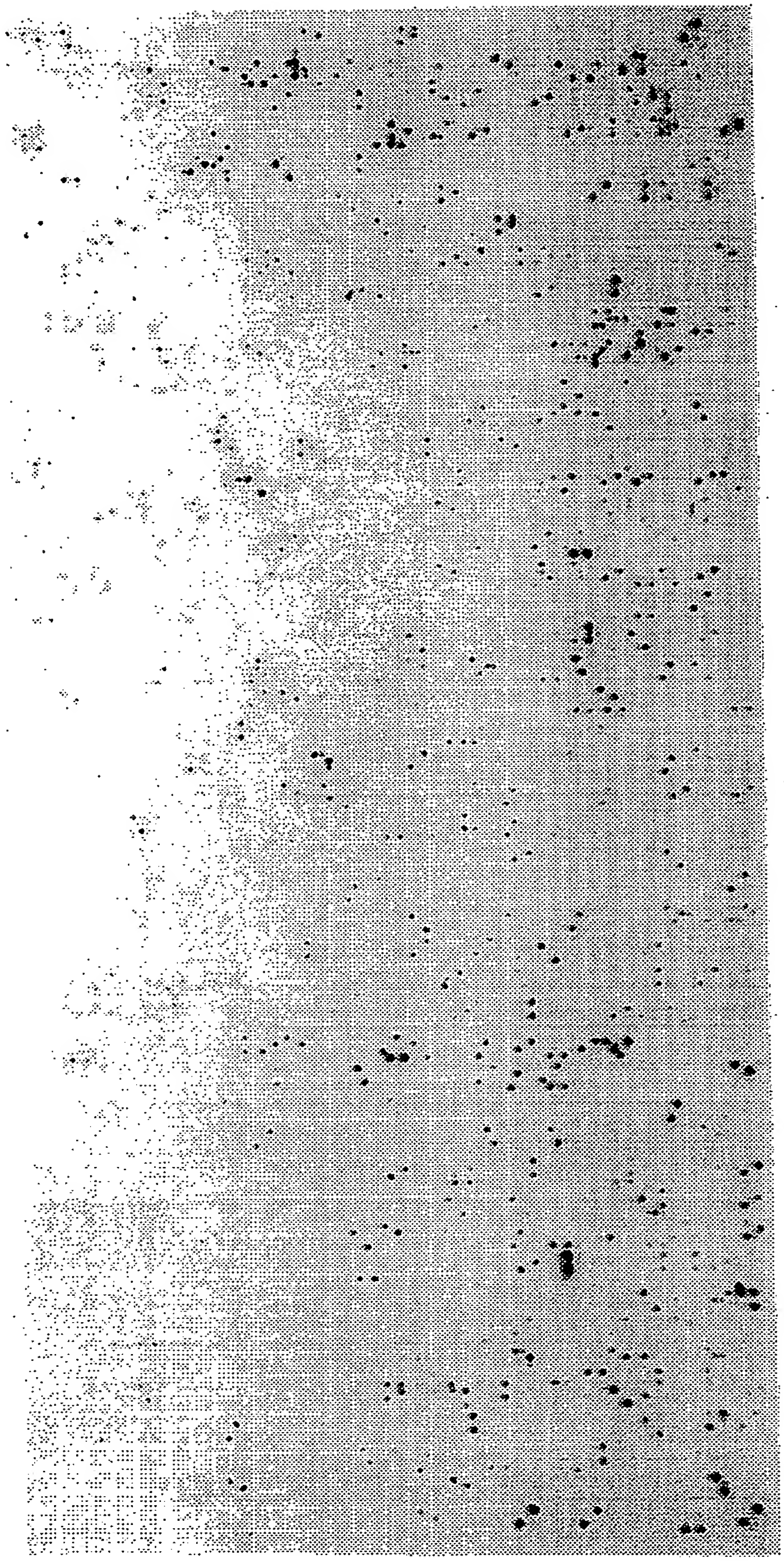


FIG. 3E

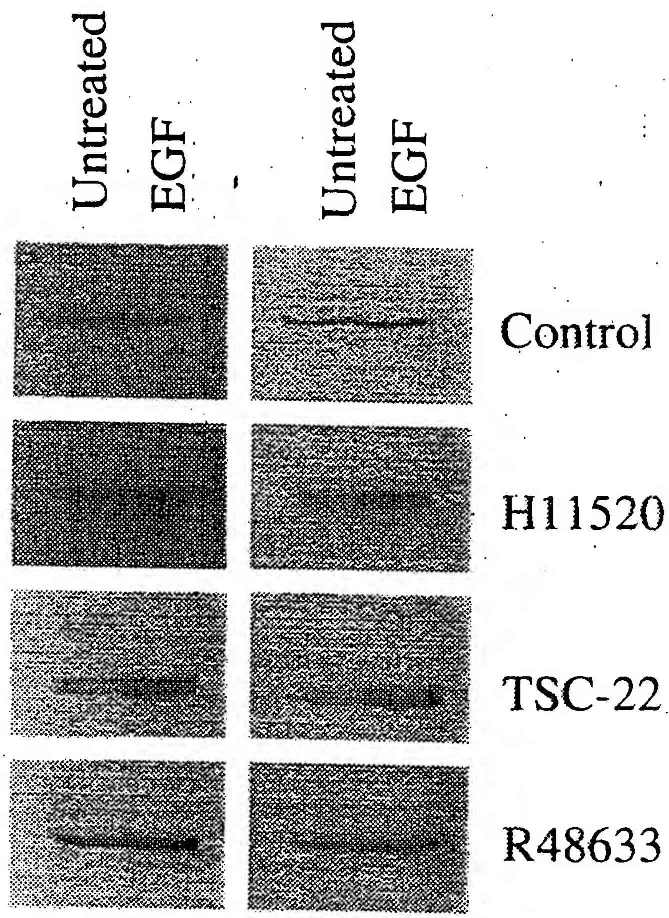
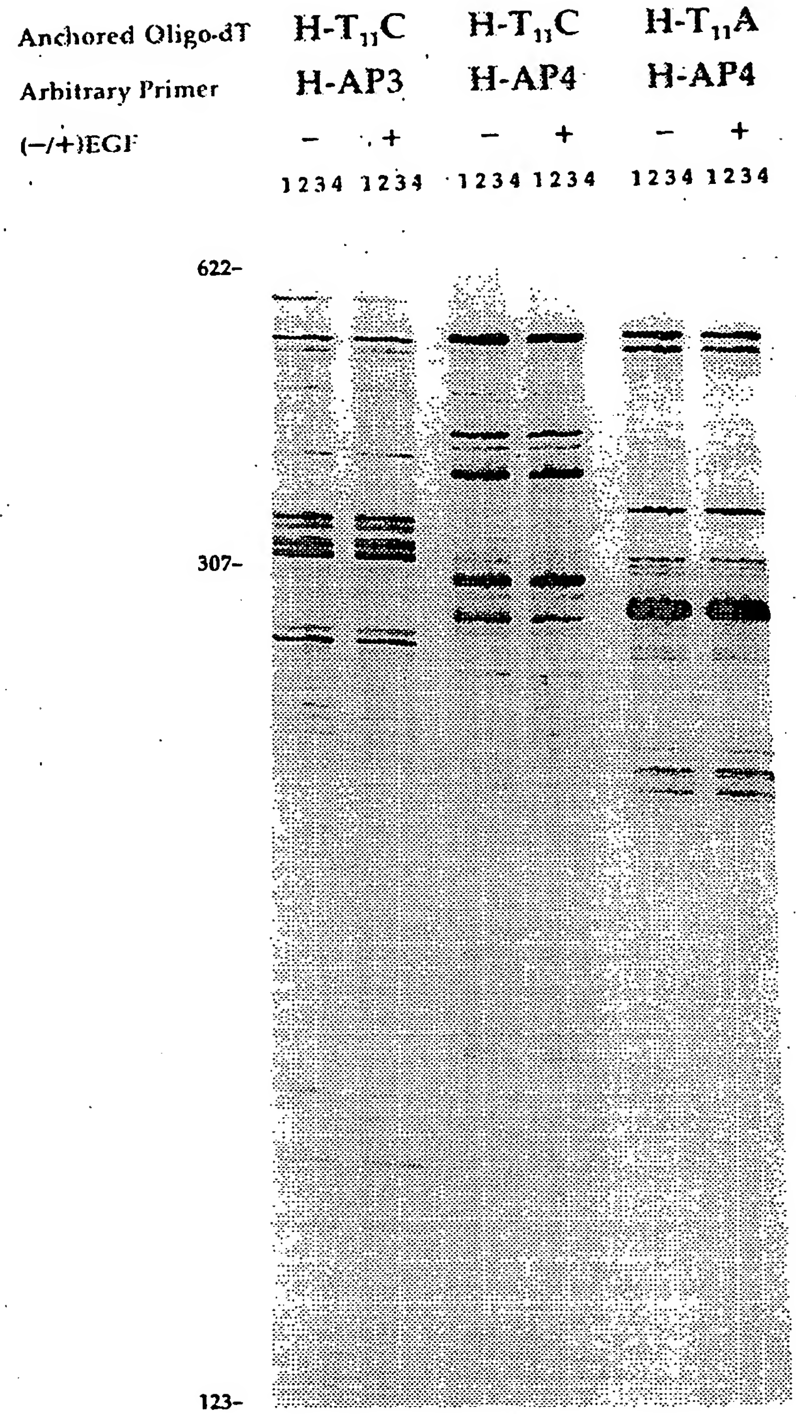



FIG. 4



Anchored Oligo-dT
H-$T_{11}$C
H-$T_{11}$C
H-$T_{11}$A
Arbitrary Primer
H-AP3
H-AP4
H-AP4
(−/+)EGF
− + − + − +
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
622-
307-
123-


FIG. 5A

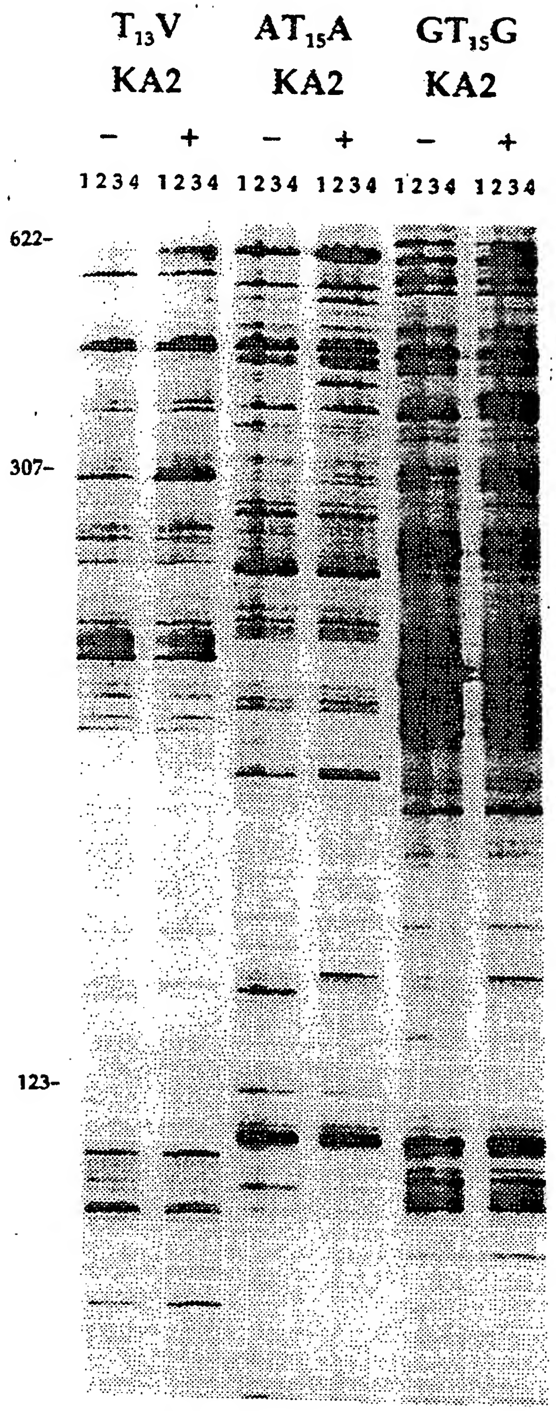

$T_{13}V$
KA2
$AT_{15}A$
KA2
$GT_{15}G$
KA2
− + − + − +
1234 1234 1234 1234 1234 1234
622-
307-
123-

FIG. 5B

FIG. 6A

FIG. 6B

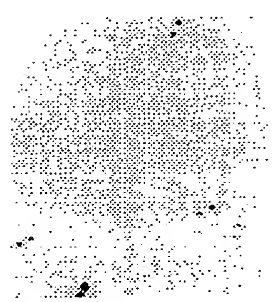
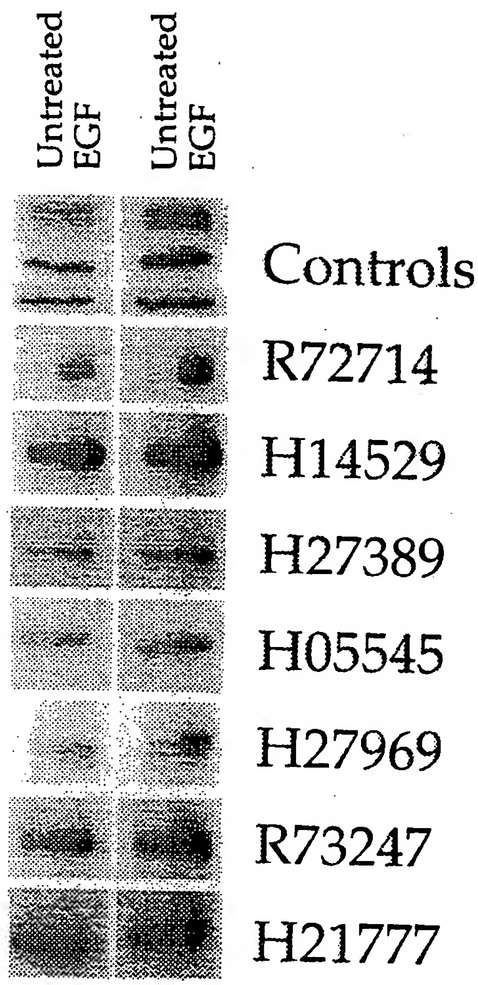


FIG. 6C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EGF
Untreated
EGF
Controls
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H27389
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R73247
H21777

FIG. 7

1 tttttttttt acaacaatgc agtcatttat ttattgagta tgtgcacatt atggtattat
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241 agccagatca aatttgtgta aaactattca gtatgtttcc cttggcttca tgtctgagga
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FIG. 8

1 acacagcccc ccgcccagcc agcatcgcag ggcttcaggg accaaccgca tagctgccta
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181 tatgccatta tctcttttcc agtattaaac actcatatgc ttatggcttn gagaaatttc
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301 agcttggcag atatctgaga aatggtttaa ttcatgctca ggagctgtgt gccttttcca
361 tcccttccgg gtcccttacc cctnacttt

FIG. 9

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FIG. 10

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481 cactggccag tcctgagcag cttgcccagt ttcaggccca gctgcagact ggctcccccc
541 ctgccaccac ccagccacag ggcaccacac agccccccgc ccagccagca tcgcagggct
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1621 tgcatatgtt tctgtgcatg ttctctacac aattgtaagg tgtcactgta tttaactgtt
1681 gcacttgtca actttcaata aagcatataa atgttgat

FIG. 11

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241 cagagttggg aaaccctcac caactgggcc tctttcacct tccacattat cccgctgcca
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FIG. 12

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421 gacaggggga

FIG. 13

```
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FIG. 14

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FIG. 15

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FIG. 16

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1141 ttgacccccc tgggggacct ggaccaggat ggctacaatg atgtggccat cggggctccc
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1441 agtgcctccc tcaccatctt ccccgccatg ttcaacccag aggagcggag ctgcagctta
1501 gaggggaacc ctgtggcctg catcaacctt agcttctgcc tcaatgcttc tggaaaacac
1561 gttgctgact ccattggttt cacagtggaa cttcagctgg actggcagaa gcagaaggga
1621 ggggtacggc gggcactgtt cctggcctcc acgcaggcaa ccctgaccca gaccctgctc
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1741 gaatttcgag acaaactctc gccgattcac atcgctctca acttctcctt ggacccccaa
1801 gccccagtgg acagccacgg cctcaggcca gccctacatt atcagagcaa gagccggata
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FIG. 17A

1921 cagctggaag tgtttgggga gcagaaccat gtgtacctgg gtgacaagaa tgccctgaac
1981 ctcactttcc atgcccagaa tgtgggtgag ggtggcgcct atgaggctga gcttcgggtc
2041 accgcccctc cagaggctga gtactcagga ctcgtcagac acccagggaa cttctccagc
2101 ctgagctgtg actactttgc cgtgaaccag agccgcctgc tggtgtgtga cctgggcaac
2161 cccatgaagg caggagccag tctgtggggt ggccttcggt ttacagtccc tcatctccgg
2221 gacactaaga aaaccatcca gtttgacttc cagatcctca gcaagaatct caacaactcg
2281 caaagcgacg tggtttcctt tcggctctcc gtggaggctc aggcccaggt caccctgaac
2341 ggtgtctcca agcctgaggc agtgctattc ccagtaagcg actggcatcc ccgagaccag
2401 cctcagaagg aggaggacct gggacctgct gtccaccatg tctatgagct catcaaccaa
2461 ggccccagct ccattagcca gggtgtgctg gaactcagct gtccccaggc tctggaaggt
2521 cagcagctcc tatatgtgac cagagttacg ggactcaact gcaccaccaa tcaccccatt
2581 aacccaaagg gcctggagtt ggatcccgag ggttccctgc accaccagca aaaacgggaa
2641 gctccaagcc gcagctctgc ttcctcggga cctcagatcc tgaaatgccc ggaggctgag
2701 tgtttcaggc tgcgctgtga gctcgggccc ctgcaccaac aagagagcca aagtctgcag
2761 ttgcatttcc gagtctgggc caagactttc ttgcagcggg agcaccagcc atttagcctg
2821 cagtgtgagg ctgtgtacaa agccctgaag atgccctacc gaatcctgcc tcggcagctg
2881 ccccaaaaag agcgtcaggt ggccacagct gtgcaatgga ccaaggcaga aggcagctat
2941 ggcgtcccac tgtggatcat catcctagcc atcctgtttg gcctcctgct cctaggtcta
3001 ctcatctaca tcctctacaa gcttggattc ttcaaacgct ccctcccata tggcaccgcc
3061 atggaaaaag ctcagctcaa gcctccagcc acctctgatg cctgagtcct cccaatttca
3121 gactcccatt cctgaagaac cagtcccccc accctcattc tactgaaaag gaggggtctg
3181 ggtacttctt gaaggtgctg acggccaggg agaagctcct ctccccagcc cagagacata
3241 cttgaagggc cagagccagg ggggtgagga gctggggatc cctccccccc atgcactgtg
3301 aaggacccct gtttacacat accctcttca tggatggggg aactcagatc cagggacaga
3361 ggcccagcct ccctgaagcc tttgcatttt ggagagtttc ctgaaacaac ttggaaagat
3421 aactaggaaa tccattcaca gttctttggg ccagacatgc cacaaggact tcctgtccag
3481 ctccaacctg caaagatctg tcctcagcct tgccagagat ccaaaagaag cccccagcta
3541 agaacctgga acttggggag ttaagacctg gcagctctgg acagccccac cctggtgggc
3601 caacaaagaa cactaactat gcatggtgcc ccaggaccag ctcaggacag atgccacaca
3661 aggatagatg ctggcccagg gccagagccc agctccaagg ggaatcagaa ctcaaatggg
3721 gccagatcca gcctggggtc tggagttgat ctggaaccca gactcagaca ttggcaccta
3781 atccaggcag atccaggact atatttgggc ctgctccaga cctgatcctg gaggcccagt

FIG. 17B

3841 tcaccctgat ttaggagaag ccaggaattt cccaggacct gaaggggcca tgatggcaac
3901 agatctggaa cctcagcctg gccagacaca ggccctccct gttccccaga gaaaggggag
3961 cccactgtcc tgggcctgca gaatttccct tctgcctgcc agctgcactg atgctgcccc
4021 tcatctctct gcccaaccct tccctcacct tggcaccaga cacccaggac ttatttaaac
4081 tctgttgcaa gtgcaataaa tctgacccag tgccccact gaccagaact ag

FIG. 17C

1 agcctgatct ctgtccaccg gtcctttata ccctcatgac ccgctgctgg gactacgacc
61 ccagtgaccg gccccgcttc accgagctgg tgtgcagcct cagtgacgtt tatcagatgg
121 agaaggacat tgccatggag caagagagga atgctcgcta ccgaaccccc aaaatcttgg
181 agcccacagc cttccaggaa cccccaccca agcccagccg acctaagtac agaccccctc
241 cgcaaaccaa cctcctgggc tccaaagctg cagttccagg ttcctgaggg tctgtgtgcc
301 agctctcctg acggcttcac cagccctatg ggagtattcc attcttcccg ttaaattcac
361 tggcacaccc cacctnttcc accgggcaca atgtntttca aaacggccac aggatggggg
421 ggagggaggg attttcattc caacccaggc aggccgagga agagggncca gcagttgttg
481 gggagg

FIG. 18

1 tttttttttt ttttgcaaat gggacaattt taattcaacc acaagtcaaa tagaaagaag
61 ttaaaagaat gtttatgcaa acacatgaga aaagaagggt gcagatgaga atgggggttg
121 gggagagaaa gaggaggagt aagaaaagag ggaaaagcaa gggaaagtaa aggaagaaag
181 agaaagaggg gcaggaagag agcggatttg gcccaaggtc ctatcttggc cgcatctctc
241 tgcttcttcc ccctgatgct tggtttgttg acaacacagc atcctgtgcc tgggactccc
301 aattagcttg ttcctgggac tgtgccccag ggtcctccct caggagggnc acatgctgtn
361 cagtccagac caaactncac attnaaataa ttt

FIG. 19

1 gaattccgtc agccctttta ctcagccaca gcctccggag ccgttgcaca cctacctgcc
61 cggccgactt acctgtactt gccgccgtcc cggctcacct ggcggtgccc gaggagtagt
121 cgctggagtc cgcgcctccc tgggactgca atgtgccgat cttagctgct gcctgagagg
181 atgtctgggg tgtccgagcc cctgagtcga gtaaagttgg gcacgttacg ccggcctgaa
241 ggccctgcag agcccatggt ggtggtacca gtagatgtgg aaaaggagga cgtgcgtatc
301 ctcaaggtct gcttctatag caacagcttc aatcctggga aaaacttcaa actggtcaaa
361 tgcactgtcc agacggagat ccgggagatc atcacctcca tcctgctgag cgggcggatc
421 gggcccaaca tccggttggc tgagtgctat gggctgaggc tgaagcacat gaagtccgat
481 gagatccact ggctgcaccc acagatgacg gtgggtgagg tgcaggacaa gtatgagtgt
541 ctgcacgtgg aagccgagtg gaggtatgac cttcaaatcc gctacttgcc agaagacttc
601 atggagagcc tgaaggagga caggaccacg ctgctctatt tttaccaaca gctccggaac
661 gactacatgc agcgctacgc cagcaaggtc agcgagggca tggccctgca gctgggctgc
721 ctggagctca ggcggttctt caaggatatg ccccacaatg cacttgacaa gaagtccaac
781 ttcgagctcc tagaaaagga agtggggctg gacttgtttt tcccaaagca gatgcaggag
841 aacttaaagc ccaaacagtt ccggaagatg atccagcaga ccttccagca gtacgcctcg
901 ctcagggagg aggagtgcgt catgaagttc ttcaacactc tcgccccgtt cgccaacatc
961 gaccaggaga cctaccgctg tgaactcatt caaggatgga acattactgt ggacctggtc
1021 attggcccta aagggatccg ccagctgact agtcaggacg caaagcccac ctgcctggcc
1081 gagttcaagc agatcaggtc catcaggtgc ctcccgctgg aggagggcca ggcagtactt
1141 cagctgggca ttgaaggtgc cccccaggcc ttgtccatca aaacctcatc cctagcagag
1201 gctgagaaca tggctgacct catagacggc tactgccggc tgcagggtga gcaccaaggc
1261 tctctcatca tccatcctag gaaagatggt gagaagcgga acagcctgcc ccagatcccc
1321 atgctaaacc tggaggcccg gcggtcccac ctctcagaga gctgcagcat agagtcagac
1381 atctacgcag agattcccga cgaaaccctg cgaaggcccg gaggtccaca gtatggcatt
1441 gcccgtgaag atgtggtcct gaatcgtatt cttggggaag gctttttggg ggaggtctat
1501 gaaggtgtct acacaaatca taaaggggag aaaatcaatg tagctgtcaa gacctgcaag
1561 aaagactgca ctctggacaa caaggagaag ttcatgagcg aggcagtgat catgaagaac
1621 ctcgaccacc cgcacatcgt gaagctgatc ggcatcattg aagaggagcc cacctggatc
1681 atcatggaat tgtatcccta tggggagctg ggccactacc tggagcggaa caagaactcc
1741 ctgaaggtgc tcaccctcgt gctgtactca ctgcagatat gcaaagccat ggcctacctg
1801 gagagcatca actgcgtgca cagggacatt gctgtccgga acatcctggt ggcctcccct
1861 gagtgtgtga agctggggga ctttggtctt tcccggtaca ttgaggacga ggactattac

FIG. 20A

1921 aaagcctctg tgactcgtct ccccatcaaa tggatgtccc cagagtccat taacttccga
1981 cgcttcacga cagccagtga cgtctggatg ttcgccgtgt gcatgtggga gatcctgagc
2041 tttgggaagc agcccttctt ctggctggag aacaaggatg tcatcggggt gctggagaaa
2101 ggagaccggc tgcccaagcc tgatctctgt ccaccggtcc tttataccct catgacccgc
2161 tgctgggact acgaccccag tgaccggccc cgcttcaccg agctggtgtg cagcctcagt
2221 gacgtttatc agatggagaa ggacattgcc atggagcaag agaggaatgc tcgctaccga
2281 acccccaaaa tcttggagcc cacagccttc caggaacccc cacccaagcc cagccgacct
2341 aagtacagac cccctccgca aaccaacctc ctggctccaa agctgcagtt ccaggttcct
2401 gagggtctgt gtgccagctc tcctacgctc accagcccta tggagtatcc atctcccgtt
2461 aactcactgc acaccccacc tctccaccgg cacaatgtct tcaaacgcca cagcatgggg
2521 gaggaggact tcatccaacc cagcagccga gaagaggccc agcagctgtg ggaggctgaa
2581 aaggtcaaaa tgcggcaaat cctggacaaa cagcagaagc agatggtgga ggactaccag
2641 tggctcaggc aggaggagaa gtccctggac cccatggttt atatgaatga taagtcccca
2701 ttgacgccag agaaggaggt cggctacctg gagttcacag ggcccccaca gaagcccccg
2761 aggctgggcg cacagtccat ccagcccaca gctaacctgg accggaccga tgacctggtg
2821 tacctcaatg tcatggagct ggtgcgggcc gtgctggagc tcaagaatga gctctgtcag
2881 ctgccccccg agggctacgt ggtggtggtg aagaatgtgg ggctgaccct gcggaagctc
2941 atcgggagcg tggatgatct cctgccttcc ttgccgtcat cttcacggac agagatcgag
3001 ggcacccaga aactgctcaa caaagacctg gcagagctca tcaacaagat gcggctggcg
3061 cagcagaacg ccgtgacctc cctgagtgag gagtgcaaga ggcagatgct gacggcttca
3121 cacaccctgg ctgtggacgc caagaacctg ctcgacgctg tggaccaggc caaggttctg
3181 gccaatctgg cccaccccacc tgcagagtga cggagggtgg gggccacctg cctgcgtctt
3241 ccgcccctgc ctgccatgta cctcccctgc cttgctgttg gtcatgtggg tcttccaggg
3301 agaaggccaa ggggagtcac cttcccttgc cactttgcac gacgccctct ccccacccct
3361 acccctggct gtactgctca ggctgcagct ggacagaggg gactctgggc tatggacaca
3421 gggtgacggt gacaaagatg gctcagaggg ggactgctgc tgcctggcca ctgctcccta
3481 agccagcctg gtccatgcag gggctcctg ggggtgggga ggtgtcacat ggtgccccta
3541 gctttatata tggacatggc aggccgattt gggaaccaag ctattccttt cccttcctct
3601 tctcccctca gatgtccctt gatgcacaga gaagctgggg aggagctttg ttttcggggg
3661 tcaggcagcc agtgagatga gggatgggcc tggcattctt gtacagtgta tattgaaatt
3721 tatttaatgt gaggtttggt ctggactgac agcatgtgcc ctcctgaggg aggaccaggg
3781 cacagtccag gaacaagcta attgggagtc caggcacagg atgctgtgtt gtcaacaaac

FIG. 20B

3841 caagcatcag ggggaagaag cagagagatg cggccaagat aggaccttgg gccaaatccg
3901 ctctcttcct gcccctcttt ctctttcttc ctttactttc ccttgctttt ccctcttttc
3961 ttactcctcc tctttctctc ccccaccccc attctcatct gcacccttct tttctcatgt
4021 gtttgcataa acattctttt aacttctttc tatttgactt gtggttgaat taaaattgtc
4081 ccatttgca

FIG. 20C

1 gacctggaga tcaacgggga gaaggtgaag ctgcagatct gggacacagc ggggcaggag
61 cgcttccgca ccatcacctc cacgtattat cgggggaccc acggggtcat ttgtggttta
121 cgacgtcacc agtgccgagt cctttntcaa cgtcaagcgg tggcttcacg aaatcaacca
181 gaactgtgat gatgtgtgcc gaatattagt gggtaataag aatgacgacc ctgagcggaa
241 ggtggtggag acggaagatg cctacaaatt cgccgggcag atgggcatcc agttgttcga
301 gaccagcgcc aaggagaatg tcaacgtggg aagagatgtt tcaactgcat tcacggagct
361 ggtcctccga gcaaagaaag acaaccttgg gcaaaacagc agcagcaaca acagaacgat
421 gttggttgaa gtttacgaag gaacattnaa cgaaagaaac gttt

FIG. 21

1 tttttttttt tttttttttt taattgtgag gaatttaatt cacttgattt ggcttcattt
61 tcttgatctg ttaaaataat cctcccatag cccccctgcc agccccatct ctgcacgaac
121 ctaccccgac ctttctgttg gaactgaaac ctgttggtgt aaatgagaag ccatggctgc
181 cctgggtttg gagctcagag gcatctagaa ggcaggacaa gaaatctgtt ggccaaaggg
241 caagacctgc cacctctgtg gaactgcagg gcctgccttg agaccaggtt ccccagctcc
301 cagaatggct gtggggacag gacaacgggg agggaaggga gctggcacag gccccggaga
361 aggggcaaga ccc

FIG. 22

```
1   gctgccggag cagcccgaag agctgcggat cgcgaggcca gtaccgaccc cgcccgcccg
61  cgcgctccgc ccccgcccgc catggcccgg gactacgacc acctcttcaa gctgctcatc
121 atcggcgaca gcggtgtggg caagagcagt ttactgttgc gttttgcaga caacactttc
181 tcaggcagct acatcaccac gatcggagtg gatttcaaga tccggaccgt ggagatcaac
241 ggggagaagg tgaagctgca gatctgggac acagcggggc aggagcgctt ccgcaccatc
301 acctccacgt attatcgggg gacccacggg gtcattgtgg tttacgacgt caccagtgcc
361 gagtcctttg tcaacgtcaa gcggtggctt cacgaaatca accagaactg tgatgatgtg
421 tgccgaatat tagtgggtaa taagaatgac gaccctgagc ggaaggtggt ggagacggaa
481 gatgcctaca aattcgccgg gcagatgggc atccagttgt tcgagaccag cgccaaggag
541 aatgtcaacg tggaagagat gttcaactgc atcacggagc tggtcctccg agcaaagaaa
601 gacaacctgg caaaacagca gcagcaacaa cagaacgatg tggtgaagct cacgaagaac
661 agtaaacgaa agaaacgctg ctgctaatgg cacccagtcc actgcagaga ctgcactgcg
721 gtccctcccc
```

FIG. 23

```
1   acagagtagc agctcagatg ccagagatcg aaagaaggct cgaatgagtg agctggaaca
61  naagtggtag atttagaaga agagaaccaa aaacttttgc tagaaaatca gcttttacga
121 gagaaaactc atggccttgt agttgagaac caggagttaa gacagcgctt ggggatggat
181 gccctggttg ctgaagagga ggcggagcaa ggggaatgaa gtnaggccan tgcgggtctg
241 ctgagtccgc agcactcaga ctacgtgcac ctctgcagca ggtgcaggcc cagttgtcac
301 cctncagaac atctccccat ggattctggc ggta
```

FIG. 24

```
1   tttttttttg ctgcattgta ccttttaatt gcatgggtag ttttaaataa atggagaaag
61  cacctttcag aagctacact agcaggaaaa aattccatca agcatttaca tagtaaattn
121 ctataatttc acaaaagatt cttgatctta ctngaagtat acatgaggga aagagccccc
181 tcagcaggtg ttcccgttgc ttacagaagn aaactaaagg acctaaaact ggaggcaagc
241 cagggtgcca aaaaggggga agagaaatga taaagaacca ttcataaatt ccatgtctac
301 ttcaaggaca tttgtctaat gacccttaca taataagtat tttaggggaa aactaccacc
361 ctttttaagg tnaaagtaca nttcttaaaa ggctggtagg tttctcaatt nt
```

FIG. 25

1 tagtctggag ctatggtggt ggtggcagcc gcgccgaacc cggccgacgg gacccctaaa
61 gttctgcttc tgtcggggca gcccgcctcc gccgccggag ccccggcggc caggctgccg
121 ctcatggtgc cagcccagag aggggccagc ccggaggcag cgagcggggg gctgccccag
181 gcgcgcaagc gacagcgcct cacgcacctg agccccgagg agaaggcgct gaggaggaaa
241 ctgaaaaaca gagtagcagc tcagactgcc agagatcgaa agaaggctcg aatgagtgag
301 ctggaacagc aagtggtaga tttagaagaa gagaaccaaa aacttttgct agaaaatcag
361 cttttacgag agaaaactca tggccttgta gttgagaacc aggagttaag acagcgcttg
421 gggatggatg ccctggttgc tgaagaggag gcggaagcca aggggaatga agtgaggcca
481 gtggccgggt ctgctgagtc cgcagcactc agactacgtg cacctctgca gcaggtgcag
541 gcccagttgt cacccctcca gaacatctcc ccatggattc tggcggtatt gactcttcag
601 attcagagtc tgatatcctg ttgggcattc tggacaactt ggacccagtc atgttcttca
661 aatgcccttc cccagagcct gccagcctgg aggagctccc agaggtctac ccagaaggac
721 ccagttcctt accagcctcc ctttctctgt cagtggggac gtcatcagcc aagctggaag
781 ccattaatga actaattcgt tttgaccaca tatataccaa gcccctagtc ttagagatac
841 cctctgagac agagagccaa gctaatgtgg tagtgaaaat cgaggaagca cctctcagcc
901 cctcagagaa tgatcaccct gaattcattg tctcagtgaa ggaagaacct gtagaagatg
961 acctcgttcc ggagctgggt atctcaaatc tgctttcatc cagccactgc ccaaagccat
1021 cttcctgcct actggatgct acagtgactg tggatacggg ggttcccttt ccccattcag
1081 tgacatgtcc tctctgcttg gtgtaaacat tcttgggagg acacttttgc caatgaactc
1141 tttccccagc tgattagtgt ctaaggaatg atccaatact gttgcccttt tccttgacta
1201 ttacactgcc tggaggatag cagagaagcc tgtctgtact tcattcaaaa agccaaaata
1261 gagagtatac agtcctagag aatccctcta tttgttcaga tctcatagat gacccccagg
1321 tattgccttt tgacatccag cagtccaagg tattgagaca tattactgga agtaagaaat
1381 attactataa ttgagaacta cagcttttaa gattgtactt ttaagattgt acttttatct
1441 taaaagggtg gtagttttcc ctaaaatact tattatgtaa gggtcattag acaaatgtct
1501 tgaagtagac atggaattta tgaatggtct ttatcatttc tcttcccccct ttttggcatc
1561 ctggcttgcc tccagtttta ggtcctttag tttgcttctg caagcaacgg gaacacctgc
1621 tgagggggct ctttccctca tgtatacttc aagtaagatc aagaatcttt tgtgaaatta
1681 tagaaattta ctatgtaaat gcttgatgga atttttcct gctagtgtag cttctgaaag
1741 gtgctttctc catttattta aaaactaccc atgcaattaa aaggtacaat gcaaaaaaaa
1801 aaaaaaaaaa attttttt

FIG. 26

```
1   aaacagtaat tctttagact ttattaaaaa atgacataaa gtgcatctta ttaaaaaatg
61  tataaaancc acataaattc cagggncccc tgtgcctggg cagtgttgat atcccttaga
121 gtggaggaag gtgagggatg gagggtgaac tggggactgg ggagaggacc agggtgcagt
181 tagttccncg tgtttgagtt caaagatgga gcgagggtgg atatggtggg aaggggcaca
241 cgggttctca cgncaacaac ggaggaaggc aggcgacagt ctcttccctg aattctgagg
301 gaaaggcgta cattgtcacg aaatctctcc tgagctcgcg ctgtcctctc
```

FIG. 27

```
1   gaaggaactg gtctgctcac acttgctggc ttgcgcatca ggactggctt tatctcctga
61  ctcacggtgc aaaggtgcac tctgcgaacg ttaagtccgt ccccagcgct tggaatccta
121 cggcccccac agccggatcc cctcagcctt ccaggtcctc aactcccgtg gacgctgaac
181 aatggcctcc atggggctac aggtaatngg catcgcgctg gccgtcctgg gctggctggc
241 cgtcatgctg tgctgcgcgc tgcccatgtg gcgcgtgacg gcctttcatc ggcagcaaca
301 ttgtcaactt gcagaccatc tgggaagggc ctattggatg aactncgtgg ttcaaaagcc
361 ngtccaagat tgnatttnaa aggttttaac gatt
```

FIG. 28

1 gaaggaactg gttctgctca cacttgctgg cttgcgcatc aggactggct ttatctcctg
61 actcacggtg caaaggtgca ctctgcgaac gttaagtccg tccccagcgc ttggaatcct
121 acggccccca cagccggatc ccctcagcct tccaggtcct caactcccgt ggacgctgaa
181 caatggcctc catggggcta caggtaatgg gcatcgcgct ggccgtcctg ggctggctgg
241 ccgtcatgct gtgctgcgcg ctgcccatgt ggcgcgtgac ggccttcatc ggcagcaaca
301 ttgtcacctc gcagaccatc tgggagggcc tatggatgaa ctgcgtggtg cagagcaccg
361 gccagatgca gtgcaaggtg tacgactcgc tgctggcact gccgcaggac ctgcaggcgg
421 cccgcgccct cgtcatcatc agcatcatcg tggctgctct gggcgtgctg ctgtccgtgg
481 tgggggggcaa gtgtaccaac tgcctggagg atgaaagcgc caaggccaag accatgatcg
541 tggcgggcgt ggtgttcctg ttggccggcc ttatggtgat agtgccggtg tcctggacgg
601 cccacaacat catccaagac ttctacaatc cgctggtggc ctccgggcag aagcgggaga
661 tgggtgcctc gctctacgtc ggctgggccg cctccggcct gctgctcctt ggcggggggc
721 tgctttgctg caactgtcca ccccgcacag acaagcctta ctccgccaag tattctgctg
781 cccgctctgc tgctgccagc aactacgtgt aaggtgccac ggctccactc tgttcctctc
841 tgctttgttc ttccctggac tgagctcagc gcaggctgtg accccaggag ggccctgcca
901 cgggccactg gctgctgggg actggggact gggcagagac tgagccaggc aggaaggcag
961 cagccttcag cctctctggc ccactcggac aacttcccaa ggccgcctcc tgctagcaag
1021 aacagagtcc accctcctct ggatattggg gagggacgga agtgacaggg tgtggtggtg
1081 gagtggggag ctggcttctg ctggccagga tagcttaacc ctgactttgg gatctgcctg
1141 catcggcgtt ggccactgtc cccatttaca ttttccccac tctgtctgcc tgcatctcct
1201 ctgttccggg taggccttga tatcacctct gggactgtgc cttgctcacc gaaacccgcg
1261 cccaggagta tggctgaggc cttgcccacc cacctgcctg ggaagtgcag agtggatgga
1321 cgggtttaga ggggaggggc gaaggtgctg taaacaggtt tgggcagtgg tgggggaggg
1381 ggccagagag gcggctcagg ttgcccagct ctgtggcctc aggactctct gcctcacccg
1441 cttcagccca gggcccctgg agactgatcc cctctgagtc ctctgcccct tccaaggaca
1501 ctaatgagcc tgggagggtg gcagggagga ggggacagct tcaccccttgg aagtcctggg
1561 gtttttcctc ttccttcttt gtggtttctg ttttgtaatt taagaagagc tattcatcac
1621 tgtaattatt attattttct acaataaatg ggacctgtgc acagg

# FIG. 29

1 aggtcctact ggaaggagtt cctggtgatg tgcacgctct ttgtgctggc cgtgctgctc
61 ccagttttat tcttgctcta ccggcaccgg aacagcatga aagtcttcct gaagcagggg
121 gaatgtgcca gcgtgcaccc caagacctgc cctgtggtgc tgccccctga gacccgccca
181 ctcaacggcc tagggcccct agcaccccgc tcgatcaccg agggtaccag tccctgtcag
241 acagcccccc ggggttcccg agtcttcact gagtcagaga agaggccact nagcatccaa
301 gacagcttcg tgggaggtat ccccagtgtg cccccggccc cgggg

# FIG. 30

```
1    gaagaaaggc tgattagaaa atttgaagct gaaaacatct ccaactacac ggcccttctg
61   ctgagccagg atggaaagac gctgtatgtg ggggcccgag aggccctctt tgcacttaac
121  agcaacctca gcttcttgcc aggcggggag taccaagagc tactgtggag tgcagatgct
181  gacaggaagc agcagtgcag cttcaagggc aaggacccaa agcgtgactg tcaaaactac
241  atcaagatcc tcctgccact caacagcagc cacctgctca cctgtggcac ggccgccttc
301  agccccctgt gtgcttacat tcacatagcg agctttactt tagcccaaga tgaggccggt
361  aatgtcattc tggaggatgg caagggtcat tgtccctttg accccaactt caagtccacg
421  gctctggtgg ttgatggtga gctgtacact ggaacagtca gtagcttcca gggaaacgac
481  ccagccattt cccggagcca gagttcccgc cccaccaaga ctgagagctc cctcaactgg
541  ctacaagacc ctgcctttgt ggcctcggct acgtcccccg agagcctggg cagccccata
601  ggtgatgatg ataagatcta cttcttcttc agcgagacgg gccaggagtt tgagttcttt
661  gagaacacca tcgtgtcccg agttgcccga gtctgtaagg gcgatgaggg tggagagcgg
721  gtgttgcagc aacgctggac ctcctttctc aaggctcagc tcctgtgctc ccggcctgat
781  gatggctttc cctttaacgt gctacaagat gtcttcaccc tgaaccccaa ccctcaggat
841  tggcgcaaga ccctttctat cggggtcttt acctcccagt ggcacagagg gaccacagaa
901  ggctctgcca tctgcgtctt caccatgaat gatgtgcaga aggcctttga cggcctgtac
961  aagaaagtaa acagagagac acagcagtgg tataccgaga cccaccaggt gcccacaccg
1021 cggccgggag cgtgcattac caacagtgcc cgggaacgga agatcaactc gtccctgcag
1081 ctcccagacc gagtgctgaa cttcctcaag gatcacttct tgatggatgg gcaggtccgc
1141 agtcgcctgc tgctgctgca gcccagagcc cgctaccagc gtgtggctgt gcaccgtgtg
1201 cctggcctgc acagcactta tgatgtccta tttctgggca ctggtgatgg ccgcctgcac
1261 aaagcagtga ccctgagctc cagagtccac atcattgagg agctgcagat cttccctcaa
1321 ggacagcctg tgcagaacct gctcttggac agccatgggg gactgttgta tgcctcctcc
1381 cattccgggg tggtgcaagt gcccgtagcc aactgcagcc tgtacccaac ctgtggagac
1441 tgcctcctgg ctcgagaccc ctactgcgcc tggactggct ctgcctgcag gctcgctagc
1501 ctctaccagc ctgatctggc ctccaggcca tggacccagg acattgaggg tgccagtgtc
1561 aaggaactct gcaagaattc ctcatacaag gcccggtttc ttgtgccagg taagccatgt
1621 aaacaagtcc agatccaacc aaacacagtg aacaccctgg cctgcccact cctctcaaac
1681 ctggccactc ggctctgggt gcacaatgga gccccagtca atgcctctgc ctcctgccgc
1741 gtgttaccca ccggggacct gctgctggtg ggcagccagc agggtttggg ggtgttccag
1801 tgttggtcga tagaagaagg attccagcag cttgtggcca gctactgccc agaggtgatg
1861 gaggaggggg taatggacca aaagaaccag cgtgatggta ccccagtcat tatcaacaca
```

FIG. 31A

1921 tcacgagtga gtgcaccggc tggtggcagg gacagctggg gtgcggacaa gtcctactgg
1981 aatgaattcc tggtgatgtg tactctgttt gtgtttgcta tggtgctttt gtttctgttc
2041 tttctctacc gacatcggga tggcatgaaa ctcttcctaa agcagggcga gtgtgccagt
2101 gtgcacccca agactcgccc tatagtgcta ccacctgaga cccgaccgct gaatggtgtc
2161 ggccctccta gcaccccact tgaccaccga ggctaccagg ctctgtcgga tagctcccca
2221 gggcccagag tcttcactga atcagagaag aggccactga gcatccagga cagctttgta
2281 gaggtgtctc ccgtgtgtcc ccggccccga gttcgactgg gctctgagat ccgagactct
2341 gtggtatgag agctgacttt agatgtggtc accctgacct cagggttgtg agtgtcagtg
2401 gaagtcagct acctctgctc tcacagaaca cag

FIG. 31B

1 gtttggcaaa aactcaagcg gctggaagga ggaagaggtt ctccagagtc ggaactgagg
61 gttggaacta tacccgggac caaactcacg gaccactcga ggcctgcaaa ccttcctggg
121 aggacaggca ggccagatgg ccgctccact ggggaatgct cccagctgtg ctgtggagag
181 aagctgatgt tttggtgtat tgtcagccat cgtccttgga ctcggagact atggcctcgc
241 tccccaccct cctcttggaa ttacaagccc tggggtttga agctgacttt atagctgcaa
301 gtgtatctcc ttttatctgg tgcctcctca aacccagtct cagacactta aatgcagaca
361 acaccttnct cctgcagaca cctgggactg agccaaggag gncttgggga aggcccttag
421 ggggagcacc ctgatgggag aggacagagc aggggttnca gca

FIG. 32

1 agaaaaagcc cantnttcac tttattggag gtctctgcct ccattcacag gagaaaggag
61 ctgggagccc catcctaagg gtcccagcat cagcccactg gagggcctgg aacagtccag
121 cactctgtgg gagaggagtg gggaggggaa tgttttagaa aaaatagatc tctatgtaca
181 tctgacatat ttatatagca cataaattag ggagtgctct gacccctgcc cgtggagccc
241 aagcactgag cagggaggtg aacgccagtc cagaaagaag gtgctgggag cccctgctct
301 gtcctctcca tccacggtgc tncccctagg g

1 agaaaaagcc cantnttcac tttattggag gtctctgcct ccattcacag gagaaaggag
61 ctgggagccc catcctaagg gtcccagcat cagcccactg gagggcctgg aacagtccag
121 cactctgtgg gagaggagtg gggaggggaa tgttttagaa aaaatagatc tctatgtaca
181 tctgacatat ttatatagca cataaattag ggagtgctct gacccctgcc cgtggagccc
241 aagcactgag cagggaggtg aacgccagtc cagaaagaag gtgctgggag cccctgctct
301 gtcctctcca tccacggtgc tncccctagg g

FIG. 33

1 cggccagata cctcagcgct acctggcgga actggatttc tctcccgcct gccggcctgc
61 ctgccacagc cggactccgc cactccggta gcctcatggc tgcaacctgt gagattagca
121 acatttttag caactacttc agtgcgatgt acagctcgga ggactccacc ctggcctctg
181 ttccccctgc tgccaccttt ggggccgatg acttggtact gaccctgagc aacccccaga
241 tgtcattgga gggtacagag aaggccagct ggttggggga acagccccag ttctggtcga
301 agacgcaggt tctggactgg atcagctacc aagtggagaa gaacaagtac gacgcaagcg
361 ccattgactt ctcacgatgt gacatggatg gcgccacccc ctgcaattgt gcccttgagg
421 agctgcgtct ggtctttggg cctctggggg accaactcca tgcccagctg cgagacctca
481 cttccagctc ttctgatgag ctcagttgga tcattgagct gctggagaag gatggcatgg
541 ccttccagga ggccctagac ccagggccct ttgaccaggg cagccccttt gcccaggagc
601 tgctggacga cggtcagcaa gccagcccct accaccccgg cagctgtggc gcaggagccc
661 cctcccctgg cagctctgac gtctccaccg cagggactgg tgcttctcgg agctcccact
721 cctcagactc cggtggaagt gacgtggacc tggatcccac tgatggcaag ctcttcccca
781 gcgatggttt tcgtgactgc aagaaggggg atcccaagca cgggaagcgg aaacgaggcc
841 ggccccgaaa gctgagcaaa gagtactggg actgtctcga gggcaagaag agcaagcacg
901 cgcccagagg cacccacctg tgggagttca tccgggacat cctcatccac ccggagctca
961 acgagggcct catgaagtgg gagaatcggc atgaaggcgt cttcaagttc ctgcgctccg
1021 aggctgtggc ccaactatgg ggccaaaaga aaaagaacag caacatgacc tacgagaagc
1081 tgagccgggc catgaggtac tactacaaac gggagatcct ggaacgggtg gatggccggc
1141 gactcgtcta caagtttggc aaaaactcaa gcggctggaa ggaggaagag gttctccaga
1201 gtcggaactg agggttggaa ctatacccgg gaccaaactc acggaccact cgaggcctgc
1261 aaaccttcct gggaggacag gcaggccaga tggcccctcc actggggaat gctcccagct
1321 gtgctgtgga gagaagctga tgttttggtg tattgtcagc catcgtcctt ggactcggag
1381 actatggcct cgcctcccca ccctcctctt ggaattacaa gccctggggt ttgaagctga
1441 ctttatagct gcaagtgtat ctccttttat ctggtgcctc ctcaaaccca gtctcagaca
1501 cttaaatgca gacaacacct tcttcctgca gacacttgga ctgagccaag gaggcttggg
1561 aggccctagg gagcaccgtg atggagagga cagagcaggg gctccagcac ttctttctgg
1621 actggcgttc acctccctgc tcagtgcttg ggctccacgg gcaggggtca gagcactccc
1681 taatttatgt gctatataaa tatgtcagat gtacatagag atctattttt tctaaaacat
1741 tcccctcccc actcctctcc cacagagtgc tggactgttc caggccctcc agtgggctga
1801 tgctgggacc cttaggatgg ggctcccagc tcctttctcc tgtgaatgga ggcagagacc
1861 tccaataaag tgccttctgg gctttttcta aaaaaaaaaa aaaaaaa

FIG. 34

1 agtactacaa gcatcattct ctcaaggaag ggttcagaac cttagataca actctgcagt
61 ttccatacaa ggagccagaa cattcagctg gacagagggg taatagagca ggcaacagct
121 tgttaagtcc aaaagtgctg ggcattgcat cgctcggtat gacttctgtg caagagatat
181 gagagagttg tccttgttga aaggagatgt ggtgaagatt tacacaaaga tgagtgcaaa
241 tggctggtgg agaggagaag taaatggcag ggtgggctgg tttccatcca catatgtggg
301 aaggaggatg aataaattca aatcccgtgt tgcaccctgc accaaaattt tcagaggaag
361 gggataatta ggaagcctgc acagcttcgt ggatttaact tgaagtgttt ttaaaaagct
421 ggcttttntg ggctgtttca acatcctccc tccttaggcc cntccta

FIG. 35

1 tttttttcc caacatgtaa ctctctcagt cttgtcagaa cacaacttct gctatggagg
61 aaatatttcc atcaggaaag ggccaagtta gtgtcttaac ttgactgcct tgaatgggga
121 ctctggaccc caggaagaat gtatttaggc tcctcacaaa aaagagtgat ggctgggcaa
181 aacaaatgta ctgcaagacc catcttccct ccagttaata cactcccagg gatgggnctg
241 cagaggggga gactctgaga gaagctggag gcccacaaaa gtccactgac cctctttctg
301 tcccagaaat gaataaagga cccagttgtg ctttccttcc aaaatcctca acaaagttgt
361 ttgtgctcca aggaaaatgt gggggantta aaaaaatcat gttcccgggt catctttgtg
421 tgtgttgcgg gggaggtngg tggggaggga aaa

FIG. 36

1 cccgccccgg cccagccgcg tcccggagcc gtcgggcatg gagccgtgga agcagtgcgc
61 gcagtggctc atccattgca aggtgctgcc caccaaccac cgggtgacct gggactcggc
121 tcaggtgttc gaccttgcgc agaccctccg cgatggagtc ctgctctgcc agctgcttaa
181 caacctccgg gcgcactcca tcaacctgaa ggagatcaac ctgaggccgc agatgtccca
241 gtttctctgt ttgaagaaca taaggacatt tctcacggcc tgttgtgaga cgtttggaat
301 gaggaaaagt gaacttttcg aggcatttga cttgtttgat gttcgtgact ttggagaggt
361 tatagaaaca ttatcacgac tttctcgaac acctatagca ttggccacag gaatcaggcc
421 cttcccaaca gaagaaagca ttaatgatga agacatctac aaaggccttc ctgatttaat
481 agatgaaacc cttgtggaag atgaagaaga tctctatgac tgtgtttatg ggaagatga
541 aggtggagaa gtctatgagg acttaatgaa ggcagaggaa gcacatcagc ccaaatgtcc
601 agaaaatgat atacgaagtt gttgtctagc agaaattaag cagacagaag aaaaatatac
661 agaaactttg gagtcaatag aaaaatattt catggcacca ctaaaaagat ttctgacagc
721 agcagaattt gattcagtat tcatcaacat tcctgaactt gtaaaacttc atcggaacct
781 aatgcaagag attcatgatt ccattgtaaa taaaaatgac cagaacttgt accaagtttt
841 tattaactac aaggaaagat tggttattta cgggcagtac tgcagtggag tggagtcagc
901 catctctagt ttagactaca tttctaagtc aaaagaagat gtcaaactga aattagagga
961 atgttccaaa agagcaaata atgggaaatt tactcttcga gacttgcttg tggttcctat
1021 gcaacgtgtt ttaaagtacc accttctcct ccaggaactg gtcaaacata ccactgatcc
1081 gactgagaag gcaaatctga aactggctct tgatgccatg aaggacttgg cacaatatgt
1141 gaatgaagtg aaaagagata atgagaccct tcgtgaaatt aaacagtttc agctatctat
1201 agagaatttg aaccaaccag ttttgctttt tggacgacct cagggagatg gtgaaattcg
1261 aataaccact ctagacaagc ataccaaaca agaaaggcat atcttcttat ttgatttggc
1321 agtgatcgta tgtaagagaa aaggtgataa ctatgaaatg aaggaaataa tagatcttca
1381 gcagtacaag atagccaata atcctacaac cgataaagaa aacaaaaagt ggtcttatgg
1441 cttctacctc atccataccc aaggacaaaa tgggttagaa ttttattgca aaacaaaaga
1501 tttaaagaag aaatggctag aacagtttga aatggctttg tctaacataa gaccagacta
1561 tgcagactcc aatttccacg acttcaagat gcataccttc actcgagtca catcctgcaa
1621 agtctgccag atgctcctga ggggaacatt ttatcaaggc tatttatgtt ttaagtgtgg
1681 agcgagagca cacaaagaat gtttgggaag agtagacaat tgtggcagag ttaattctgg
1741 tgaacaaggg acactcaaac taccagagaa acggaccaat ggactgcgaa gaactcctaa
1801 acaggtggat ccaggtttac caaagatgca ggtcattagg aactattctg gaacaccacc
1861 cccagctctg catgaaggac ccccttttaca gctccaggcc ggggataccg ttgaacttct

FIG. 37A

1921 gaaaggagat gcacacagtc tgttttggca gggcagaaat ttagcatctg gagaggttgg
1981 atttttcca agtgatgcag tcaagccttg cccatgtgtg cccaaaccag tagattattc
2041 ttgccaaccc tggtatgctg gagcaatgga aagattgcaa gcagagaccg aacttattaa
2101 tagggtaaat agtacttacc ttgtgaggca caggaccaaa gagtcaggag aatatgcaat
2161 tagcattaag tacaataatg aagcaaagca catcaagatt ttaacaagag atggcttttt
2221 tcacattgca gaaaatagaa aatttaaaag tttaatggaa cttgtggagt actacaagca
2281 tcattctctc aaggaagggt tcagaacctt agatacaact ctgcagtttc catacaagga
2341 gccagaacat tcagctggac agaggggtaa tagagcaggc aacagcttgt taagtccaaa
2401 agtgctgggc attgccatcg ctcggtatga cttctgtgca agagatatga gagagttgtc
2461 cttgttgaaa ggagatgtgg tgaagattta cacaaagatg agtgcaaatg gctggtggag
2521 aggagaagta aatggcaggg tgggctggtt tccatccaca tatgtggaag aggatgaata
2581 aattcaaatc ccgtgttgca ccctgcacca aaaatttcag agaagggata aatagaagcc
2641 tgcacagcat cgtgaattaa ctgaagtgtt taaaaagctg catttctggc tgttcaacat
2701 cctccctcct tagcccctcc taagtcttaa tgctgagatt tctaaagatg ctggtactga
2761 cagattaatg gcttgcctag agctgtgcaa gaaacagcct gccagtctgt cattgtcagg
2821 gaccagggca aaaccaagag ctgttcttcc cagaagagcc ctgcaaacac attggttcgt
2881 gcttcccttt acttcttctg gtcagatacc atgaatgcca gtcatcagta aatcttaata
2941 cactttgct ttattctcac atgccattca ccagattatt tgatggtaca aagaagcaga
3001 agtgtaattt tccttttccc agcatgacga aaaattggag ttctgccatt tgagcagctt
3061 actggagaga tccagcctta cttgtcttaa attgtccaac aaggtgactc attgcccggc
3121 aaacactttt accctcagat gttactcatg atattataaa atatgaggcc agtgctcagg
3181 tttgcatcat aagtgagcta tccctgaagg gttttaatta cttatttggt gtcctgatta
3241 tatttgcaaa cttctttata aaaggtgaaa aaagcacaca aaagagaggg tgtcttcata
3301 ttaaaccttc acaaccttca tgatttcata ggattatttt ggaaatatag cacttgactt
3361 tatgaaagga tctgggctag gtatattagg ggtagttgcc aataacctga agaagctggc
3421 attgtttaca gaaacagatc aagggctata atttatgtca ttttatagca gcagtatcta
3481 ttaatacatg ccttttcctc ccatccacct cccccgcaca cacacaaaga tgacctggga
3541 catgattttt ttattcccac attttcttgg agcacaaaca actttgttga ggatttgga
3601 aggaaagcac aactgggtcc tttattcatt tctgggacag aaagagggtc agtggacttt
3661 tgtgggcctc cagcttctct cagagtctcc ccctctgcag cccatcctgg gagtgtatta
3721 actggaggga agatgggtct tgcagtacat ttgttttgcc cagccatcac tctttttgt
3781 gaggagccta aatacattct tcctggggtc cagagtcccc attcaaggca gtcaagttaa

FIG. 37B

3841 gacactaact tggccctttc ctgatggaaa tatttcctcc atagcagaag ttgtgttctg
3901 acaagactga gagagttaca tgttgggaaa aaaaagaagc attaacttag tagaactgaa
3961 ccaggagcat taagttctga aattttgaat catctctgaa atgaagcagg tgtagcctgc
4021 cctctcatca atccgtccgt ctgggtgcca gaactcaagg ttcagtggac acatccccct
4081 gttagagacc ctcatgggct aggacttttc atctaggata gattcaagac ctttacctca
4141 gaattatgta aactgtgatt gtgttttaga aaaattatta tttgctaaaa ccatttaagt
4201 ctttgtatat gtgtaaatga tcacaaaaat gtattttata aaatgttctg tacaataaag
4261 ttacacctca aagtgtactc ttggaatgga ttctttcctg taaagtctta tctgcgactc
4321 tgtctcggga atgttttgtc tgttgccgtc agccgaactt tgttatggag ggagcagcct
4381 cacacaagca gaaacactcc tgtggatggt attgtagcat gtattgttta ttttagtcaa
4441 tagaccctct ccttataaat ggtgtttagt cttcctgttg catttcatgg gcctgggggt
4501 ttcctrgcag aggatattgg agccccttt tgtgacatta ccaattacat ctttgtccac
4561 gtttaatact ttgttttgga aaatttaaat gctgcagatt tgtgtagagt tctaatacca
4621 aagacagaag taaatgtttt ccatatactt tgtcttgcct gtatgcagcc cttgtgtaat
4681 atggtgaatt agagtggtat ttcactttgt attattttgt aaatatgtca atataataaa
4741 tagtgactaa aaaaaaaaaa aa

FIG. 37C

1 tttttactt tattttcgtt ttaattttt ggaaggatat acaccacata tcccatgggc
61 aataaagcgc attcaatgtn tttataagcc aaacagtcac tttgtttaag caaacacaag
121 tacaaagtaa aatagaacca caaaataatg aactgcatgt tcataacata caaaaatcgc
181 cgcctactca gtaggtaact acaacattcc aactccngaa tatatttata aatttacatt
241 ttcagttaaa aaantagact tttgagagtt cagattttgt tttagatttt gttttcttac
301 attctggaga ncccgaagct ncagctcagc ccctcttccc ttattttgct ccccaaagcc
361 ttccccccaa atcancactg ncctgncccc cctntaaggg cttagaggtg agcatntccc
421 ct

FIG. 38

1 ccgcagaact tggggagccg ccgccgccat ccgccgccgc agccagcttc cgccgccgca
61 ggaccggccc ctgccccagc ctccgcagcc gcggcgcgtc cacgcccgcc cgcgcccagg
121 gcgagtcggg gtcgccgcct gcacgcttct cagtgttccc cgcgccccgc atgtaacccg
181 gccaggcccc cgcaacggtg tcccctgcag ctccagcccc gggctgcacc cccccgcccc
241 gacaccagct ctccagcctg ctcgtccagg atggccgcgg ccaaggccga gatgcagctg
301 atgtccccgc tgcagatctc tgacccgttc ggatcctttc ctcactcgcc caccatggac
361 aactacccta agctggagga gatgatgctg ctgagcaacg gggctcccca gttcctcggc
421 gccgccgggg ccccagaggg cagcggcagc aacagcagca gcagcagcag cgggggcggt
481 ggaggcggcg gggcggcag caacagcagc agcagcagca gcaccttcaa ccctcaggcg
541 gacacgggcg agcagcccta cgagcacctg accgcagagt cttttcctga catctctctg
601 aacaacgaga aggtgctggt ggagaccagt taccccagcc aaaccactcg actgcccccc
661 atcacctata ctggccgctt ttccctggag cctgcaccca acagtggcaa caccttgtgg
721 cccgagcccc tcttcagctt ggtcagtggc ctagtgagca tgaccaaccc accggcctcc
781 tcgtcctcag caccatctcc agcggcctcc tccgcctccg cctcccagag cccacccctg
841 agctgcgcag tgccatccaa cgacagcagt cccatttact cagcggcacc caccttcccc
901 acgccgaaca ctgacatttt ccctgagcca caaagccagg ccttcccggg ctcggcaggg
961 acagcgctcc agtacccgcc tcctgcctac cctgccgcca agggtggctt ccaggttccc
1021 atgatccccg actacctgtt tccacagcag cagggggatc tgggcctggg caccccagac
1081 cagaagccct tccagggcct ggagagccgc acccagcagc cttcgctaac ccctctgtct
1141 actattaagg cctttgccac tcagtcgggc tcccaggacc tgaaggccct caataccagc
1201 taccagtccc agctcatcaa acccagccgc atgcgcaagt atcccaaccg gcccagcaag
1261 acgccccccc acgaacgccc ttacgcttgc ccagtggagt cctgtgatcg ccgcttctcc
1321 cgctccgacg agctcacccg ccacatccgc atccacacag gccagaagcc cttccagtgc
1381 cgcatctgca tgcgcaactt cagccgcagc gaccacctca ccacccacat ccgcacccac
1441 acaggcgaaa agcccttcgc ctgcgacatc tgtggaagaa agtttgccag gagcgatgaa
1501 cgcaagaggc ataccaagat ccacttgcgg cagaaggaca agaaagcaga caaaagtgtt
1561 gtggcctctt cggccacctc ctctctctct tcctacccgt ccccggttgc tacctcttac
1621 ccgtccccgg ttactacctc ttatccatcc ccggccacca cctcataccc atcccctgtg
1681 cccacctcct tctcctctcc cggctcctcg acctacccat cccctgtgca cagtggcttc
1741 ccctccccgt cggtggccac cacgtactcc tctgttcccc ctgctttccc ggcccaggtc
1801 agcagcttcc cttcctcagc tgtcaccaac tccttcagcg cctccacagg gctttcggac
1861 atgacagcaa ccttttctcc caggacaatt gaaatttgct aaagggaaag gggaaagaaa

FIG. 39A

1921 gggaaaaggg agaaaaagaa acacaagaga cttaaaggac aggaggagga gatggccata
1981 ggagaggagg gttcctctta ggtcagatgg aggttctcag agccaagtcc tccctctcta
2041 ctggagtgga aggtctattg gccaacaatc ctttctgccc acttcccctt ccccaattac
2101 tattcccttt gacttcagct gcctgaaaca gccatgtcca agttcttcac ctctatccaa
2161 agaacttgat ttgcatggat tttggataaa tcatttcagt atcatctcca tcatatgcct
2221 gaccccttgc tcccttcaat gctagaaaat cgagttggca aaatggggtt tgggcccctc
2281 agagccctgc cctgcacccct tgtacagtgt ctgtgccatg gatttcgttt ttcttggggt
2341 actcttgatg tgaagataat ttgcatattc tattgtatta tttggagtta ggtcctcact
2401 tgggggaaaa aaaaaaaaaa aagccaagca aaccaatggt gatcctctat tttgtgatga
2461 tgctgtgaca ataagtttga accttttttt ttgaaacagc agtcccagta ttctcagagc
2521 atgtgtcaga gtgttgttcc gttaaccttt ttgtaaatac tgcttgaccg tactctcaca
2581 tgtggcaaaa tatggtttgg tttttctttt ttttttttga aagtgttttt tcttcgtcct
2641 tttggtttaa aaagtttcac gtcttggtgc cttttgtgtg atgccccttg ctgatggctt
2701 gacatgtgca attgtgaggg acatgctcac ctctagcctt aaggggggca gggagtgatg
2761 atttgggggga ggctttggga gcaaaataag gaagagggct gagctgagct tcggttctcc
2821 agaatgtaag aaaacaaaat ctaaaacaaa atctgaactc tcaaaagtct atttttttaa
2881 ctgaaaatgt aaatttataa atatattcag gagttggaat gttgtagtta cctactgagt
2941 aggcggcgat ttttgtatgt tatgaacatg cagttcatta ttttgtggtt ctattttact
3001 ttgtacttgt gtttgcttaa acaaagtgac tgtttggctt ataaacacat tgaatgcgct
3061 ttattgccca tgggatatgt ggtgtatatc cttccaaaaa attaaaacga aaataaagta
3121 gctgcgattg gg

FIG. 39B

1 ttaaggtata cacttttatt caactggtct caagtcagtg tacaggtaag ccctggctgc
61 ctccacccac tcccagggag accaaaagcc ttcatacatc tcaagttggg ggacaaaaaa
121 gggggaaggg ggggcacgaa ggctcatcat tcaaaataaa acaaaataaa aaagtattaa
181 ggcgaagatt aaaaaaattt tgcattacat aatttacacg aaagcaatgc tatcacctcc
241 cctgtgtgga cttgggagag gactgggcca ttctccttag gagagaagtg ggggtgggct
301 tttagggatg ggcaagggga ctttcctgtt aacaacggca tcttcatatt ttgggaattg
361 actntttaaa aaaaaccaac aatgtggcaa ttcaaagtcc ntcgggccac atttgtggaa
421 ctttnggggg gttgctcgnt cccacccgac tgttgttcac cttt

FIG. 40

1 gcccagcacc ccaaggcggc caacgccaaa actctccctc ctcctcttcc tcaatctcgc
61 tctcgctctt ttttttttc gcaaaaggag gggagagggg gtaaaaaaat gctgcactgt
121 gcggcgaagc cggtgagtga gcggcgcggg gccaatcagc gtgcgccgtt ccgaaagttg
181 cctttatgg ctcgagcggc cgcggcggcg ccctataaaa cccagcggcg cgacgcgcca
241 ccaccgccga gaccgcgtcc gcccgcgagc acagagcctc gcctttgccg atccgccgcc
301 cgtccacacc cgccgccagg taagcccggc cagccgaccg gggcatgcgg ccgcggccct
361 tcgcccgtgc agagccgccg tctgggccgc agcgggggc gcatggggcg gaaccggacc
421 gccgtggggg gcgcgggaga agcccctggg cctccggaga tgggggacac cccacgccag
481 ttcgcaggcg cgaggccgcg ctcgggcggg cgcgctccgg gggtgccgct ctcggggcgg
541 gggcaaccgg cggggtcttt gtctgagccg ggctcttgcc aatggggatc gcacggtggg
601 cgcggcgtag cccccgtcag gcccggtggg ggctggggcg ccatgcgcgt gcgcgctggt
661 cctttgggcg ctaactgcgt gcgcgctggg aattggcgct aattgcgcgt gcgcgctggg
721 actcaatggc gctaatcgcg cgtgcgttct ggggcccggg cgcttgcgcc acttcctgcc
781 cgagccgctg gcgcccgagg gtgtggccgc tgcgtgcgcg cgcgcgaccc ggtcgctgtt
841 tgaaccgggc ggaggcgggg ctggcgcccg gttgggaggg ggttggggcc tggcttcctg
901 ccgcgcgccg cggggacgcc tccgaccagt gtttgccttt tatggtaata acgcggccgg
961 cccggcttcc tttgtcccca atctgggcgc gcgccggcgc cccctggcgg cctaaggact
1021 cggcgcgccg gaagtggcca gggcgggggc gacttcggct cacagcgcgc ccggctattc
1081 tcgcagctca ccatggatga tgatatcgcc gcgctcgtcg tcgacaacgg ctccggcatg
1141 tgcaaggccg gcttcgcggg cgacgatgcc ccccgggccg tcttcccctc catcgtgggg
1201 cgccccaggc accaggtagg ggagctggct gggtggggca gccccgggag cgggcgggag
1261 gcaagggcgc tttctctgca caggagcctc ccggtttccg ggtgggctg cgcccgtgct
1321 cagggcttct tgtcctttcc ttcccagggc gtgatggtgg gcatgggtca gaaggattcc
1381 tatgtgggcg acgaggccca gagcaagaga ggcatcctca ccctgaagta ccccatcgag
1441 cacggcatcg tcaccaactg ggacgacatg gagaaaatct ggcaccacac cttctacaat
1501 gagctgcgtg tggctcccga ggagcacccc gtgctgctga ccgaggcccc cctgaacccc
1561 aaggccaacc gcgagaagat gacccaggtg agtggcccgc tacctcttct ggtggccgcc
1621 tccctccttc ctggcctccc ggagctgcgc cctttctcac tggttctctc ttctgccgtt
1681 ttccgtagga ctctcttctc tgacctgagt ctcctttgga actctgcagg ttctatttgc
1741 tttttcccag atgagctctt tttctggtgt ttgtctctct gactaggtgt ctgagacagt
1801 gttgtgggtg taggtactaa cactggctcg tgtgacaagg ccatgaggct ggtgtaaagc
1861 ggccttggag tgtgtattaa gtaggcgcac agtaggtctg aacagactcc ccatcccaag

FIG. 41A

1921 accccagcac acttagccgt gttctttgca ctttctgcat gtcccccgtc tggcctggct
1981 gtccccagtg gcttccccag tgtgacatgg tgcatctctg ccttacagat catgtttgag
2041 accttcaaca ccccagccat gtacgttgct atccaggctg tgctatccct gtacgcctct
2101 ggccgtacca ctggcatcgt gatggactcc ggtgacgggg tcacccacac tgtgcccatc
2161 tacgaggggt atgccctccc ccatgccatc ctgcgtctgg acctggctgg ccgggacctg
2221 actgactacc tcatgaagat cctcaccgag cgcggctaca gcttcaccac cacggccgag
2281 cgggaaatcg tgcgtgacat taaggagaag ctgtgctacg tcgccctgga cttcgagcaa
2341 gagatggcca cggctgcttc cagctcctcc ctggagaaga gctacgagct gcctgacggc
2401 caggtcatca ccattggcaa tgagcggttc cgctgccctg aggcactctt ccagccttcc
2461 ttcctgggtg agtggagact gtctcccggc tctgcctgac atgagggtta cccctcgggg
2521 ctgtgctgtg gaagctaagt cctgccctca tttccctctc aggcatggag tcctgtggca
2581 tccacgaaac taccttcaac tccatcatga agtgtgacgt ggacatccgc aaagacctgt
2641 acgccaacac agtgctgtct ggcggcacca ccatgtaccc tggcattgcc gacaggatgc
2701 agaaggagat cactgccctg gcacccagca caatgaagat caaggtgggt gtctttcctg
2761 cctgagctga cctgggcagg tcagctgtgg ggtcctgtgg tgtgtgggga gctgtcacat
2821 ccagggtcct cactgcctgt ccccttccct cctcagatca ttgctcctcc tgagcgcaag
2881 tactccgtgt ggatcggcgg ctccatcctg gcctcgctgt ccaccttcca gcagatgtgg
2941 atcagcaagc aggagtatga cgagtccggc ccctccatcg tccaccgcaa atgcttctag
3001 gcggactatg acttagttgc gttacaccct ttcttgacaa aacctaactt gcgcagaaaa
3061 caagatgaga ttggcatggc tttatttgtt ttttttgttt tgttttggtt tttttttttt
3121 ttttggcttg actcaggatt taaaaactgg aacggtgaag gtgacagcag tcggttggag
3181 cgagcatccc ccaaagttca caatgtggcc gaggactttg attgcattgt tgtttttttta
3241 atagtcattc caaatatgag atgcattgtt acaggaagtc ccttgccatc ctaaaagcca
3301 ccccacttct ctctaaggag aatggcccag tcctctccca agtccacaca ggggaggtga
3361 tagcattgct ttcgtgtaaa ttatgtaatg caaaattttt ttaatcttcg ccttaatact
3421 tttttatttt gttttatttt gaatgatgag ccttcgtgcc ccccctcccc ccttttttgtc
3481 ccccaacttg agatgtatga aggcttttgg tctccctggg agtgggtgga ggcagccagg
3541 gcttacctgt acactgactt gagaccagtt gaataaaagt gcacacctta aaaatgaggc
3601 caagtgtgac tttgtggtgt ggctgggttg ggggcagcag agggtg//

FIG. 41B

1 ctcgatttng ggaagttgta gactgcacaa ttaaaacaga tccagtcact nggagatcaa
61 gaggatttgg atttgtgctt ttcaaagatg ctgctagtgt tgataaggtt ttggaactna
121 aagaacacaa actggatggc aaattgatag atcccaaaag ggccaaagct ttaaaggga
181 aagaacctcc caaaaaggtt tttgtgggtg gattgagccc ggatacttct gaagaacaaa
241 ttaaagnata ttttggagcc tttggagaga ttgaaaatat tgaacttccc atggatacaa
301 naacaaattg aanggaag

FIG. 42

1 gatctcttcc gccgccattt taaatccagc tccatacaac gctccgccgc cgctgctgcc
61 gcgacccgga ctgcgcgcca gcacccccct gccgacagct ccgtcactat ggaggatatg
121 aacgagtaca gcaatataga ggaattcgca gagggatcca agatcaacgc gagcaagaat
181 cagcaggatg acggtaaaat gtttattgga ggcttgagct gggatacaag caaaaaagat
241 ctgacagagt acttgtctcg atttggggaa gttgtagact gcacaattaa aacagatcca
301 gtcactggga gatcaagagg atttggattt gtgcttttca aagatgctgc tagtgttgat
361 aaggttttgg aactgaaaga acacaaactg gatggcaaat tgatagatcc caaaagggcc
421 aaagctttaa aagggaaaga acctcccaaa aaggtttttg tgggtggatt gagcccggat
481 acttctgaag aacaaattaa agaatatttt ggagcctttg gagagattga aaatattgaa
541 cttcccatgg atacaaaaac aaatgaaaga agaggatttt gttttatcac atatactgat
601 gaagagccag taaaaaaatt gttagaaagc agataccatc aaattggttc tgggaagtgt
661 gaaatcaaag ttgcacaacc caaagaggta tataggcagc aacagcaaca acaaaaaggt
721 ggaagaggtg ctgcagctgg tggacgaggt ggtacgaggg gtcgtggccg aggtcagggc
781 caaaactgga accaaggatt taataactat tatgatcaag gatatggaaa ttacaatagt
841 gcctatggtg gtgatcaaaa ctatagtggc tatggcggat atgattatac tgggtataac
901 tatgggaact atggatatgg acagggatat gcagactaca gtggccaaca gagcacttat
961 ggcaaggcat ctcgaggggg tggcaatcac caaaacaatt accagccata ctaaaggaga
1021 acattggaga aaacaggagg agatgttaaa gtaacccatc ttgcaggacg acattgaaga
1081 ttggtcttct gttgatctaa gatgattatt ttgtaaaaga ctttctagtg tacaagacac
1141 cattgtgtcc aactgtatat agctgccaat tagttttctt tgtttttact ttgtcctttg
1201 ctatctgtgt tatgactcaa tgtggatttg tttatacaca ttttatttgt atcatttcat
1261 gttaaacctc aaataaatgc ttccttatgt g

FIG. 43

```
1    gaattcgcag agggatccaa gatcaacgcg agcaagaatc agcaggatga cggtaaaatg
61   tttattggag gcttgagctg ggatacaagc aaaaaagatc tgacagagta cttgtctcga
121  tttggggaag ttgtagactg cacaattaaa acagatccag tcactgggag atcaagagga
181  tttggatttg tgcttttcaa agatgctgct agtgttgata aggttttgga actgaaagaa
241  cacaaactgg atggcaaatt gatagatccc aaaagggcca aagctttaaa agggaaagaa
301  cctcccaaaa aggtttttgt gggtggattg agcccggata cttctgaaga acaaattaaa
361  gaatattttg gagcctttgg agagattgaa aatattgaac ttcccatgga tacaaaaaca
421  aatgaaagaa gaggattttg ttttatcaca tatactgatg aagagccagt aaaaaaattg
481  ttagaaagca gataccatca aattggttct gggaagtgtg aaatcaaagt tgcacaaccc
541  aaagaggtat ataggcagca acagcaacaa caaaaaggtg gaagaggtgc tgcagctggt
601  ggacgaggtg gtacgagggg tcgtggccga ggtcagggcc aaaactggaa ccaaggattt
661  aataactatt atgatcaagg atatggaaat tacaatagtg cctatggtgg tgatcaaaac
721  tatagtggct atggcggata tgattatact gggtataact atgggaacta tggatatgga
781  cagggatatg cagactacag tggccaacag agcacttatg gcaaggcatc tcgagggggt
841  ggcaatcacc aaaacaatta ccagccatac taaaggagaa cattggagaa aacaggagga
901  gatgttaaag taacccatct tgcaggacga cattgaagat tggtcttctg ttgatctaag
961  atgattattt tgtaaaagac tttctagtgt acaagacacc attgtgtcca actgtatata
1021 gctgccaatt agttttcttt gtttttactt tgtcctttgc tatctgtgtt atgactcaat
1081 gtggatttgt ttatacacat tttatttgta tcatttcatg ttaaacctca aataaatgct
1141 tccttatgtg attgcttttc tgcgtcaggt actacatagc tctgtaaaaa atgtaattta
1201 aaataagcaa taattaaggc acagttgatt ttgtagagta ttggtccata cagagaaact
1261 gtggtccttt ataaatagcc agccagcgtc accctcttct ccaatttgta ggtgtatttt
1321 atgctcttaa ggcttcatct tctccctgta actgagattt ctaccacacc tttgaacaat
1381 gttctttccc ttctggttat ctgaagactg tcctgaaagg aagacataag tgttgtgatt
1441 agtagaagct ttgtaatcat aacacaatga gtaattcttg tataaaagtt cagatacaaa
1501 aggagcactg taaaactggt aggagctatg gtttaagagc attggaagta gttacaactc
1561 aaggattttg gtagaaaggt atgagtttgg tcgaaaaatt aaaatagtgg caaaataaga
1621 tttagttgtg ttttctcaga gccgccacaa gattgaacaa aatgttttct gtttgggcat
1681 cctgaggaag ttgtattagc tgttaatgct ctgtgagttt agagaaaagt cttgatagta
1741 aatctagttt ttgacacagt gcatgaacta agtagttaaa tatttacata ttcagaaagg
1801 aatagtggaa aaggtatctt ggttatgaca aagtcattac aaatgtgact aagtcattac
1861 aaatgtgact gagtcattac agtggaccct ctgggtgcat tgaaaagaat ccgttttata
```

FIG. 44A

1921 tccaggtttc agaggacctg gaataataat aagctttgga ttttgcattc agtgtagttg
1981 gattttggga ccttggcctc agtgttattt actgggattg gcatacgtgt tcacaggcag
2041 agtagttgat ctcacacaac gggtgatctc acaaaactgg taagtttctt atgctcatga
2101 gccctccctt ttttttttta atttggtgcc tgcaactttc ttaacaatga ttctacttcc
2161 tgggctatca cattataatg ctcttggcct ctttttgct gctgttttgc tattcttaaa
2221 cttaggccaa gtaccaatgt tggctgttag aagggattct gttcattcaa catgcaactt
2281 tagggaatgg aagtaagttc atttttaagt tgtgtggtca gtaggtgcgg tgtctagggt
2341 agtgaatcct gtaagttcaa atttatgatt aggtgacgag ttgacattga gattgtcctt
2401 ttcccctgat caaaaaaatg aataaagcct ttttaaacg

FIG. 44B

1 ttttacagat ctttttgact atcttcctct cactgccttg gtggatgggc agatcttctg
61 tctacatggt ggtctctcgc catctataga tacactggat catatcagag cacttgatcg
121 cctacaagaa gttccccatg agggtccaat gtgtgacttg ctgtggtcag atccagatga
181 ccgtggtggt tggggtatat ctcctcgagg agctggttac acctttgggc aagatatttc
241 tgagacattt aatcatgcca atggcctcac gttggtgtct agagctcacc agctagtgat
301 ggagggatat aactggtgcc atgaccggaa tgtagtaacg attttcagtg ctccaaacta
361 ttgttatcgt tgtggtaacc aagctgcaat catgggaact tgacgatact ctaaaatact
421 ctttcntgca gttttgaccc agcanctcgt agggccgag

FIG. 45

1 gagagctcgg ctctcggagg aggaggcgca cggccagcgg cagtactgcg gtgagagcca
61 gcggccagcg ccacgctcaa cagccgccag aagtacacga ggaaccggcg gcggcgtgtg
121 cgtgtaagcc ggcggcggcg cgggaggagc cggagcggca gccggctggg gcgggtggca
181 tcatggacga gaaggtgttc accaaggagc tggaccagtg gatcgagcag ctgaacgagt
241 gcaagcagct gtccgagtcc caggtcaaga gcctctgcga gaaggctaaa gaaatcctga
301 caaaagaatc caacgtgcaa gaggttcgat gtccagttac tgtctgtgga gatgtgcatg
361 ggcaatttca tgatctcatg gaactgttta gaattggtgg caaatcacca gatacaaatt
421 acttgtttat gggagattat gttgacagag gatattattc agttgaaaca gttacactgc
481 ttgtagctct taaggttcgt taccgtgaac gcatcaccat tcttcgaggg aatcatgaga
541 gcagacagat cacacaagtt tatggtttct atgatgaatg tttaagaaaa tatggaaatg
601 caaatgtttg gaaatatttt acagatcttt ttgactatct tcctctcact gccttggtgg
661 atgggcagat cttctgtcta catggtggtc tctcgccatc tatagataca ctggatcata
721 tcagagcact tgatcgccta caagaagttc cccatgaggg tccaatgtgt gacttgctgt
781 ggtcagatcc agatgaccgt ggtggttggg gtatatctcc tcgaggagct ggttacacct
841 ttgggcaaga tatttctgag acatttaatc atgccaatgg cctcacgttg gtgtctagag
901 ctcaccagct agtgatggag ggatataact ggtgccatga ccggaatgta gtaacgattt
961 tcagtgctcc aaactattgt tatcgttgtg gtaaccaagc tgcaatcatg gaacttgacg
1021 atactctaaa atactctttc ttgcagtttg acccagcacc tcgtagaggc gagccacatg
1081 ttactcgtcg taccccagac tacttcctgt aatgaaattt taaacttgta cagtattgcc
1141 atgaaccata tatcgaccta atggaaatgg gaagagcaac agtaactcca aagtgtcaga
1201 aaatagttaa cattcaaaaa acttgttttc acatggacca aaagatgtgc catataaaaa
1261 tacaaagcct cttgtcatca acagccgtga ccactttaga atgaaccagt tcattgcatg
1321 ctgaagcgac attgttggtc aagaaaccag tttctggcat agcgctattt gtagttactt
1381 ttgtttctct gagagactgc agataataag atgtaaacat taacacctcg tgaatacaat
1441 ttaacttcca tttagctata gctttactca gcatgactgt agataaggat agcagcaaac
1501 aatcattgga gcttaatgaa catttttaaa aataattacc aaggcctccc ttctacttgt
1561 gagttttgaa attgttcttt ttattttcag ggataccgtt taatttaatt atatgatttg
1621 tctgcactca gtttattccc tactcaaatc tcagccccat gttgttcttt gttattgtca
1681 gaacctggtg agttgttttg aacagaactg tttttccccc ttcctgtaag acgatgtgac
1741 tgcacaagag cactgcagtg tttttcataa taaacttgtg aactaac

FIG. 46

```
1   gtttacagat gccacttagt tacactggtt ttnnttttttc agtctcatct gggttgganc
61  caaagacatt cagaggcatg gnaagaggca aagcatcaga catctcattg gnggcaggta
121 cttccngact actgtaccac ctgctgtatc cttccccacc tcancacccc caaagccatt
181 tagngccaaa tgctacagta aaaacccaat gcatttacat aaaanaatgc ctaactgcat
241 attnacattt ttnagaaaaa aaatcccatt angctcttct agaaagttat ggcaggaaag
301 gtaaggncca aggctntgag caagccatnt gtggnaactt aaagtagatg agcactgagt
361 ttctccatag ttggaaaaaa ngccacactg agcccncttt tcccgtggag ggcaagntga
421 gnccctccnt ttataccccg ttgagatntc ag
```

FIG. 47

```
1   gagaaaaggg ttggggagaa gcctctgcag tcctggaaga tgtggggttc tgggtgagag
61  gcatcagccc cacaagtatg tttttgtgtc ttaagatagc agtttacttt gaaaaagtga
121 aaaaggcttc cgggctgtcc tctgcccagt gagatggagg acgctagaga aagtgctgag
181 tgtcccgaga gaggcccccg agccagtgca tggnaggtcc ttcggcctgg ntcagctngg
241 ctgcaggatg cccactttga gga
```

FIG. 48

1 cccgcgggca ggggcggcga gtgcgcgggc cgccgccctt ctcggcgggc agcgcgcgag
61 gaccaggccg aggaggaagt ggcggcggcg gcggcgggct ccccgcccga ggaggaagat
121 gcagaccttt ctgaaaggga agagagttgg ctactggctg agcgagaaga aaatcaagaa
181 gctgaatttc caggctttcg ccgagctgtg caggaagcga gggatggagg ttgtgcagct
241 gaaccttagc cggccgatcg aggagcaggg cccccctggac gtcatcatcc acaagctgac
301 tgacgtcatc cttgaagccg accagaatga tagccagtcc ctggagctgg tgcacaggtt
361 ccaggagtac atcgatgccc accctgagac catcgtcctg gacccgctcc ctgccatcag
421 aaccctgctt gaccgctcca agtcctatga gctcatccgg aagattgagg cctacatgga
481 agacgacagg atctgctcgc caccccttcat ggagctcacg agcctgtgcg gggatgacac
541 catgcggctg ctggagaaga acggcttgac tttcccattc atttgcaaaa ccagagtggc
601 tcatggcacc aactctcacg agatggctat cgtgttcaac caggagggcc tgaacgccat
661 ccagccaccc tgcgtggtcc agaatttcat caaccacaac gccgtcctgt acaaggtgtt
721 cgtggttggc gagtcctaca ccgtggtcca gaggccctca ctcaagaact tctccgcagg
781 cacatcagac cgtgagtcca tcttcttcaa cagccacaac gtgtcaaagc cggagtcgtc
841 atcggtcctg acggagctgg acaagatcga gggcgtgttc gagcggccga gcgacgaggt
901 catccgggag ctctcccggg ccctgcggca ggcactgggc gtgtcactct tcggcatcga
961 catcatcatc aacaaccaga cagggcagca cgccgtcatt gacatcaatg ccttcccagg
1021ctacgagggc gtgagcgagt tcttcacaga cctcctgaac cacatcgcca ctgtcctgca
1081 gggccagagc acagccatgg cagccacagg ggacgtggcc ctgctgaggc acagcaagct
1141 tctggccgag ccggcgggcg gcctggtggg cgagcggaca tgcaacgcca gccccggctg
1201 ctgcggcagc atgatgggcc aggacgcgcc ctggaaagct gaggccgacg cgggcggcac
1261 cgccaagctg ccgcaccaga gactcggctg caacgccggc gtgtctccca gcttccagca
1321 gcattgtgtg gcctccctgg ccaccaaggc ctcctcccag tagccacgga gccgggaccc
1381 agagggcagc gcaggcgcag gagcacaccc gctgggccag cagctcccaa cggcgatgct
1441 actactaaga atccccagtg atctgattct tctgtttttt aatttttaac ctgattttct
1501 gatgtcatga tctaaatgag ggtagaaga gagtaccagg tggtccaccg ttggggagcg
1561 gggccgtccg cctgctctct actgtgcaga cctcctaact gagtttacac acgcttgtgt
1621 tgcaacacta ggtctggatg ggaggtgagg ggggtgcgta tactgccatg ccagtgtctg
1681 tgcacatccc tgtctgttgt ctccatggcc actgtggact gggacccttg aagcctgccc
1741 atgtgggtgt gggaggctga tcagtgcgtg tgagagtggc ttcccttctg cctgactccc
1801 cactccctga cctgcccctt ccttgttttt cctcctactg gtctccacca aggctttgtt
1861 agccccccacc ctgcctggtg tgcagctaac ccctccctcc ccacagccag aggaggccac

FIG. 49A

1921 agacccctca gggagttccg cgctggggtc tgggctgtgc tccctcacta aagggaagga
1981 aaggaagctg ggcgtcctcc gggcccccca acacacgtcc catttagccc tgcacagcgg
2041 tctccttccc ctaagccagc actgctgctc cctggagccg ggaaggaggc tgcctggctg
2101 gaggccgagc cgatgggcct gtgctgagga tttgtgctgt gatttgggca aatcattcca
2161 ggtctttggg cctccacccc ctcgtctcta gtggacattt gagatcagag agcaccacag
2221 ggctggcttt gtgccctaac ccctgggatg cagcctgcct ttccataaag tcacctaggt
2281 gaggataggc gcgggagcct cggcatgaca ccatggagat cggggccctc ttcccagtgg
2341 gttcactcct tttcacacct gctgggtccc tcctcgccca gcaggcctgg tccacctctc
2401 attgcaagcc cgcaagcact gagccgagta aggtgcttag tgtgagccac ccgcccccca
2461 tagcttctgc acacctcaga ctcaccccat caccttggca gcaaagcact gctctgccgt
2521 ctgacccctg atccaggcag cagccccctc cgcagagaaa agggttgggg agaagcctct
2581 gcagtcctgg aagatgtggg gtgctgggtg agaggcatca gcccccacaa gtatgttttt
2641 gtgtcttaag atagcagttt actttgaaaa agtgaaaaag gcttccgggc tgtcctctgc
2701 ccagtgagat ggaggacgct agagaaagtg ctgagtgtcc cgagagaggc ccccgagcca
2761 gtgcatggag gtcttcggcc tggctcagct gggctgcagg atgcccactt tgaggaggga
2821 ggcacagggc ttgggcgagg ggcagaggcc atcagaactg cccggctttt ttggaaactg
2881 aggacccaac aactaaccac gtttacacga cttgagtttt gaaccccgat taatgtctgt
2941 acgtcacctt tcctagttct gaccctgagc cctggggaac aggaaagcgt ggctggcctc
3001 ttgcactgct ttgtctccaa aataaactac tgaaatcaaa ccgcatttc

FIG. 49B

1 ggttgagccc tacaactgca tcctcaccac ccacaccacc ctggagcact ctgattgtgc
61 cttcatggta gacaatgagg ccatctatga catctgtcgt agaaacctcg atatcgagcg
121 cccaacctac accaacctta accgccttat tagccagatt gtgtcctcca tcactgcttc
181 cctgagattt gatggagncc tgaatgttga cctgacagaa ttccagacca acctgggtgc
241 cctacccccg catccacttn cctctggcca catatgcccc tgtcatctct gctgagaang
301 cctaccacga acagcttact gtagtagaga tcaccaatgc ttgntttgag ccagccaacc
361 agatggtgaa atntgganct ttgncattgg taaattacat ggggtttgcn gtctgtt

FIG. 50

```
1    tgtcggggac ggtaaccggg acccgtgctc tgctcctgtc gccttcgcct cctgaatccc
61   tagccatatg cgtgagtgca tctccatcca cgttggccag gctggtgtcc agattggcaa
121  tgcctgctgg gagctctact gcctggaaca cggcatccag cccgatggcc agatgccaag
181  tgacaagacc attgggggag gagatgactc cttcaacacc ttcttcagtg agacgggcgc
241  tggcaagcac gtgccccggg ctgtgtttgt agacttggaa cccacagtca ttgatgaagt
301  tcgcactggc acctaccgcc agctcttcca ccctgagcag ctcatcacag gcaaggaaga
361  tgctgccaat aactatgccc gagggcacta caccattggc aaggagatca ttgaccttgt
421  gttggaccga attcgcaagc tggctgacca gtgcacccgt cttcagggct tcttggtttt
481  ccacagcttt ggtgggggaa ctggttctgg gttcacctcc ctgctcatgg aacgcctgtc
541  agttgattat ggcaagaaat ccaagctgga gttctccatt tacccggcac cccaggtttc
601  cacagctgta gttgagccct acaactccat cctcaccacc cacaccaccc tggagcactc
661  tgattgtgcc ttcatggtag acaatgaggc catctatgac atctgtcgta gaaacctcga
721  tatcgagcgc ccaacctaca ctaaccttaa ccgccttatt agccagattg tgtcctccat
781  cactgcttcc ctgagatttg atggagccct gaatgttgac ctgacagaat tccagaccaa
841  cctggtcccc taccccccgca tccacttccc tctggccaca tatgcccctg tcatctctgc
901  tgagaaagcc taccatgaac agctttctgt agcagacatc accaatgctt gctttgagcc
961  agccaaccag atggtgaaat gtgaccctgg ccatggtaaa tacatggctt gctgcctgtt
1021 gtaccgtggt gacgtggttc ccaaagatgt caatgctgcc attgccacca tcaaaaccaa
1081 gcgcacgatc cagtttgtgg attggtgccc cactggcttc aaggttggca tcaactacca
1141 gcctcccact gtggtgcctg gtggagacct ggccaaggta cagagagctg tgtgcatgct
1201 gagcaacacc acagccattg ctgaggcctg ggctcgcctg gaccacaagt ttgacctgat
1261 gtatgccaag cgtgcctttg ttcactggta cgtgggtgag gggatggagg aaggcgagtt
1321 ttcagaggcc cgtgaagata tggctgccct tgagaaggat tatgaggagg ttggtgtgga
1381 ttctgttgaa ggagagggtg aggaagaagg agaggaatac taattatcca ttcctttgg
1441 ccctgcagca tgtcatgctc ccagaatttc agcttcagct taactgacag atgttaaagc
1501 tttctggtta gattgtttc acttggtgat catgtctttt ccatgtgtac ctgtaatatt
1561 tttccatcat atctcaaagt aaagtcatta acatca
```

FIG. 51

1 ctgtgaccca gaagtcttcg aattcactgg tttttcagac tctgccacgg cacatgcgac
61 gaagagccat gagccacaac gtcaaacgcc ttcccagacg gttacaggag attgcccaga
121 aagaggcgga gaaagccgta catcagaaaa aagaacattc aaaaaataaa tgccataaag
181 ctcgaagatg tcacatgaac cggacgctag aatttaaccg tagacaaaag aagaacattt
241 ggttagaaac tcacatctgg cacgccaagc ggtttcatat ggtcaagaag tggggctact
301 gccttgggga gaggccaaca gtcaagagcc acagagcctg ctatcgagcc atgacgaacc
361 ggtgcctcct gcaggattta tcctattact gttgtttgga gttgaaaggc aaagaggaag
421 aaatactaaa ggcgctttct ggaatgtgta acatagacac agggctgacg tttgcagcag
481 ttcactgctt gtctggaaag cgccaaggga gccttgtgct ttatcgggtg aataaatatc
541 ccagagaaat gcttgggcct gttacgttta tctggaagtc ccagaggacc ccgggtgacc
601 cttctgagag caggcagctg tggatctggc tgcatccaac ccttaaacag gatatcttag
661 aggaaataaa agcagcgtgc cagtgtgtgg aacccatcaa atcagctgtc tgcatcgctg
721 acccacttcc aacaccatcc caagaaaaaa gccaaactga attgcctgac gagaaaattg
781 gcaagaaaag aaaaaggaaa gatgatggag aaaatgctaa accaattaaa aaaattatcg
841 gtgatggaac tagagatcca tgtctaccat actcttggat ctctccaacc acaggcatta
901 taatcagcga tttgacgatg gagatgaaca gattccggct gattgggcca ctttcccact
961 ccatcctaac tgaagcaata aaagctgctt ctgtccacac tgtgggagag gacacagagg
1021 agacacctca ccgctggtgg atagaaacct gtaagaaacc tgacagcgtt tcccttcatt
1081 gcagacaaga agccattttc gagttgttgg gaggaataac atcaccagca gaaattccgg
1141 caggtactat tctgggactg acagttgggg atcctcgaat aaatttgccc caaaagaagt
1201 ccaaagcttt gcccaatcca gaaaaatgcc aagataatga gaaagttaga cagctgcttc
1261 tggagggtgt gcctgtggaa tgtacgcata gctttatctg gaaccaagat atctgtaaga
1321 gtgtcacaga gaataaaatc tcggatcagg atttaaaccg gatgaggagt gaattgctgg
1381 tgcctgggtc acagcttatt ttaggtcccc atgaatccaa gatacctata cttttgattc
1441 agcagccagg aaaagtgact ggtgaagatc gactaggctg gggaagtggc tgggatgtcc
1501 tactcccaaa gggctggggc atggctttct ggattccatt tatttatcga ggtgtgagag
1561 tcggagggtt gaaagagtct gcagtgcatt ctcagtataa gaggtcgcct aatgtcccag
1621 gcgattttcc agactgccct gccgggatgc tgtttgcgga agagcaagct aagaatcttc
1681 ttgaaaagta caaaagacgc cctcctgcaa aacggcccaa ctacgttaag cttggcactc
1741 tggcaccttt ctgctgtccc tgggagcagt taactcaaga ctgggagtca agagtccagg
1801 cttacgaaga accttctgta gcttcatctc caaatggtaa ggagagtgac ctaagaagat
1861 ctgaggtgcc ttgtgctccc atgcctaaaa aaactcatca gccatctgat gaagtgggca
1921 catccataga gcaccccagg gaggcagagg aggtaatgga tgcagggtgt caagaatcgg

FIG. 52A

1981 cagggcctga gaggatcaca gaccaggagg ccagtgaaaa ccatgttgct gccacaggga
2041 gtcacctctg cgttctcagg agtagaaaat tactgaagca actgtcagcc tggtgtgggc
2101 ccagttctga ggatagtcgg ggaggccggc gagctcccgg cagaggccag caaggattga
2161 ccagagaggc ttgcctgtcc atcttgggcc acttccccag ggccctggtt tgggtcagcc
2221 tgtccctgct cagcaagggc agccccgagc ctcacaccat gatctgtgtc ccagccaagg
2281 aggacttcct ccagctccat gaggactggc attactgtgg gccccaggaa tccaaacaca
2341 gtgacccatt caggagcaag atcctgaaac agaaagagaa gaagaaaagg gagaagaggc
2401 agaagccagg acgtgcctct tctgatggcc cggcggggga agagcccgtg gctgggcagg
2461 aagctctgac tctagggctg tggtcaggcc ctctgccgcg tgtgacgttg cactgctcca
2521 gaactctcct aggcttgtg actcagggag atttttccat ggctgttggc tgtggagaag
2581 ccctggggtt tgttagcttg acaggcttgc tggatatgct gtccagccag cctgcagcgc
2641 agaggggctt agtgctactg aggcctcccg cctctctgca gtatcgattt gcgaggattg
2701 ctattgaggt gtgaatgcgt gcttgtatcc cagcagggca tagataatac gttattattg
2761 tctgccaagt tctacatgtg gagaatctgc ttctgcttta aaatatcatg tgaaactccc
2821 tggaaacaag aataaaaaat tatgtattat gcagatgatg aaatgtttac atcattccag
2881 taatgtcatt gattttcatc tttccctgtc cttgctgtaa tacttttaaa ttatttggcc
2941 aaaagctttg tattatgatc tcttggtctg tgtagttgtg gctgaaaata atgagaagct
3001 ctacgagtta tcatcccctt tttttgttag aaacaaaggg cttgtcaggt ctatttgaaa
3061 aacctcatag tcatgtgata agcaacaata gatgtttaat gatttcactg ttatagcaga
3121 agacaagaga agacgcttgg cctctgtaca tgaaatatgg gctcctgatg gacctcattc
3181 aattctgtac tgtgatttcc atgccgaaca actcaagcct taaagagaga aatcatggac
3241 aactgatttc tgcctgtttt caggcaggca cagtttatgg cgtcagtgct aggctggaat
3301 tagaaagtgg gggtctatga cgtggacttc ctgactcttt gatctctttg ttgttgacca
3361 acacttgatc ctactagtta cttaattttt ttaagtaaaa aattattatt attttgtttc
3421 tgcaaagatt ttctcaaagc catagaggag catttctcag aatatgttct atgatatgtg
3481 tcacctaaaa aagtaagaga ttccaaggtc aggttgatat ggaaactcta ggttaaataa
3541 agttaagcat ttctttatga aagaacttct ggaaacttcc atgtgataat gtgcattgcg
3601 gatctctagg aaggaaatga tagtgtatag tattttctaa atacttgtga ttcctaaagt
3661 tctcttacaa ggagcccttt gtaggaccag tgttcttagt agcgcgcttt gggcagtgtg
3721 gctgtgtagt gcatagctac ctctgcaagg tgataactaa gccggcaagc tgcctttcaa
3781 cactcatgca gtcacgttgt ccacctgaga ttctcaacag ggtataaaag gaaggtctca
3841 tcttgcctca caggaagagt gggctcagtg tggctttttt ccaactatgg agaaactcag
3901 tgctcatcta ctttaagttt ccacatatgg cttgctcata gccttggtcc ttacctttcc

FIG. 52B

3961 tgccataact ttctagaaga gcttaatggg atttttttct aaaaaatgta aatatgcagt
4021 taggcattat tttatgtaaa tgcattgggt ttttactgta gcatttggca ctaaatggct
4081 ttgggggtga tgaggtgggg aaggatacag caggtggtac agtagtcagg aagtacctgc
4141 caccaatgag atgtctgatg ctttgcctct taccatgcct ctgaatgtct ttggatccaa
4201 cccagatgag actgaaaaaa aaaaaacagt gtaactaagt ggcatctgta aacagaataa
4261 atgaaaatgt cacctg

FIG. 52C

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09119

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 96 41893 A (THE UNIVERSITY OF TENNESSEE RESEARCH CORPORATION) 27 December 1996 (1996-12-27)<br>page 3, line 26 -page 4, line 34; claims 12-34; examples 1,2,3A,4<br>page 18, line 29 -page 22, line 23<br>--- | 1,7,8, 13,14, 16-18, 21-25, 29,44, 45, 47-49, 52-56, 60,61, 67,78, 79, 85-88, 90-93 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 November 1999 | 03. 12.99 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Luzzatto, E |

International Application No
PCT/US 99/09119

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 97 22720 A (BEATTIE K.L.) 26 June 1997 (1997-06-26)<br>page 4, line 25 -page 11, line 19; figure 5<br>page 14, line 6 -page 16, line 8<br>page 19, line 10 -page 21, line 12; claims | 1,2,7,8, 13-18, 21-25, 60,85-88 |
| X | G. PIETU ET AL.: "Novel gene transcripts preferentially expressed in human muscles revealed by quantitative hybridisation of a high density cDNA array"<br>GENOME RESEARCH,<br>vol. 6, 1996, pages 492-503, XP000597086<br>US<br>cited in the application<br>abstract<br>page 493, column 1, line 39 - line 54<br>page 496, column 1, line 51 -page 500, column 1, line 25 | 1,7,13, 14, 16-18, 21-26, 85-88 |
| X | K. KWOK WONG ET AL.: "Stress-inducible gene of Salmonella typhimurium identified by arbitrarily primed PCR of RNA"<br>PROCEEDINGS OFTHE NATIONAL ACADEMY OF SCIENCES USA,<br>vol. 91, January 1994 (1994-01), pages 639-643, XP002122208<br>US | 79,80, 85-88, 90-93 |
| A | the whole document | 1,60,67, 74 |
| A | C.E.LOPEZ-NIETO ET AL.: "Selective amplification of protein-coding regions of large sets of genes using statistically designed primer sets"<br>NATURE BIOTECHNOLGY,<br>vol. 14, July 1996 (1996-07), pages 857-861, XP002090066<br>UK<br>cited in the application | 29 |
| A | J. WELSH ET AL.: "Arbitrarily primed PCR fingerprinting of RNA"<br>NUCLEIC ACIDS RESEARCH,<br>vol. 20, no. 19, 1992, pages 4965-4970, XP000508271<br>UK<br>cited in the application<br>the whole document | 1-8,13, 14, 16-18, 20-28, 60-71, 73-83, 85-89 |
| A | WO 97 27317 A (AFFIMETRIX,INC.) 31 July 1997 (1997-07-31)<br>the whole document | 1 |

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09119

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | CAETANO-ANOLLES, G.: "Scanning of nucleic acids by in vitro amplification: New developments and applications" NATURE BIOTECHNOLOGY, vol. 14, December 1996 (1996-12), pages 1668-1674, XP002122510 UK page 1672, column 1, line 26 -column 2, line 17 | 1,29 |
| P,X | T. TRENKLE ET AL.: "Non-stoichiometric reduced complexity probes for cDNA arrays" NUCLEIC ACIDS RESEARCH, vol. 26, no. 17, September 1998 (1998-09), pages 3883-3891, XP002122209 UK the whole document | 1-83, 85-94 |

In... national application No.
PCT/US 99/09119

# INTERNATIONAL SEARCH REPORT

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 84,95
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 84,95

Claims 84 and 95 relate to a set of nucleic acid molecules each molecule of the set being a portion of a longer molecule, whose length is comprised from about 300 nt to 4276 nt. No indication of the length of the claimed portion is to be found either in the claim or in the descritpion. The said claims are thus unsearchable.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/09119

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9641893 A | 27-12-1996 | US 5962221 A | 05-10-1999 |
| | | AU 6272896 A | 09-01-1997 |
| WO 9722720 A | 26-06-1997 | AU 1687597 A | 14-07-1997 |
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| | | EP 0880598 A | 02-12-1998 |

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